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Jason Anthony Miranda

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The Dissertation Committee for Jason Anthony Miranda Certifies that this is the approved version of the following dissertation:

Sex differences and hormone influences on auditory processing of communication signals in the green treefrog, *Hyla cinerea*

Committee:

Walter Wilczynski, Co-Supervisor

Michael J. Ryan, Co-Supervisor

Creagh W. Breuner

Harold H. Zakon

Dennis McFadden

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by

Jason Anthony Miranda, B.S.

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Sex differences and hormone influences on auditory processing of communication signals in the green treefrog, *Hyla cinerea*

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Jason Anthony Miranda, Ph. D.

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Supervisors: Walter Wilczynski & Michael J. Ryan

In animal communication, individuals within a species often vary widely in their behavioral responses to species-typical signals. These variations in behavior may be due to differences in the sensory processing of communication signals. Sensory processing of behaviorally relevant stimuli is likely to be influenced by reproductive hormones. Here I report investigations on the influence of sex and reproductive condition on auditory processing in the green treefrog, *Hyla cinerea*.

I conducted electrophysiological experiments that tested how sex and reproductive condition influence the neural representation of sounds in the auditory midbrain, the torus semicircularis. I found differences between and within the sexes that were both frequency-dependent (low vs. high frequency) and stimulus-dependent (tones vs. calls). For sex differences at auditory threshold, females were less sensitive to frequencies outside the spectral range of the male advertisement call and were not

different from males inside the range. Sex differences were also stimulus-dependent with females more sensitive to the advertisement call than males. For stimuli consistent with close-range communication, I tested whether or not sex differences in response strengths to advertisement call and noise stimuli depended on the reproductive state of the female. I found that in response to low frequency stimuli postmated females had significantly reduced response strengths compared to males and unmated females. Additionally, I tested whether circulating reproductive hormones influenced auditory processing by manipulating androgen levels and assessing neural thresholds and response strengths to auditory stimuli. Elevated androgen levels in females resulted in increased thresholds and reduced response strengths but only in response to stimuli that are consistent with species-typical communication. Together the evidence from these studies suggest that sex and reproductive hormones influence auditory processing in a way that shapes the filtering properties of the auditory system for the detection of communication signals.

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INTRODUCTION

To understand the neural mechanisms that result in communication related behavior, we must consider how sex and reproductive condition influence the sensory processing of communication signals. In many auditory communication systems, a given signal is intended for both male and female receivers. The same stimulus evokes a different behavioral response in each sex. For example, males and females can show differences in behavioral discrimination among variants of a signal. Females may respond to a species-typical male vocalization but not to a similar sounding heterospecific vocalization. In contrast, males may respond equally to both stimuli. One hypothesis to explain such differences predicts that male and female auditory systems process these stimuli differently. Discrimination can be based on various spectral or temporal components of the stimulus, and the sexes may differ in the components that they use. For this reason, we might expect to see sex differences in auditory processing, particularly in relation to conspecific signals. More specifically, sex differences may be limited to particular spectral bands within the hearing range of the species (frequency-dependent). Additionally, differences may be limited to a particular type of stimulus

such as a species-typical auditory signal but not to simple stimuli such as pure tones, white noise or clicks.

Females also show condition-dependent behavioral responses based on their reproductive state. Levels of responsiveness typically increase as oviposition/ovulation approaches and reduce after oviposition/ovulation. Neural systems related to behavioral motivation and reproductive regulation have received a great deal of attention in relation to variation in behavior. Since these neural systems rely on afferent input from auditory processing areas, the possibility remains that the auditory system may function differently across reproductive conditions and result in behavioral differences. Just as for sex differences, condition-dependent auditory processing may be both frequency-dependent and stimulus-dependent.

In this review, I will focus on several animal communication systems for which sex differences and/or condition-dependent differences in behavior have been related to differences in auditory processing. I will summarize some common principles that occur across taxa to help guide future directions in research on the neural mechanisms of communication behavior.

FISH

Evidence from a vocalizing teleost demonstrates that steroid hormones influence auditory processing of a communication signal in a frequency-dependent manner. In the vocalizing plainfin midshipman, *Porichthys notatus*, females perform phonotaxis to male advertisement hums only during the summer breeding season. The male advertisement

hum is a very simple signal consisting of a fundamental frequency of 90-100 Hz, upper harmonics to about 400 Hz and no temporal modulation of the envelope shape (reviewed in Bass et al., 2005). Experiments have shown that females will approach a speaker broadcasting a single tone within the natural fundamental frequency range (McKibben & Bass, 1998). The simplicity of this signal and the female response to it have allowed for clear associations between the neural encoding of the stimulus in the auditory periphery and its behavioral salience across reproductive conditions in females. Females mate once during the breeding season and after depositing their eggs, do not respond to male hums (McKibben & Bass, 1998). This seasonal difference in behavior is associated with an increase in the maximum stimulus frequency at which *P. notatus* eighth nerve afferents effectively encode the stimulus (Sisneros & Bass, 2003a). Best excitatory frequencies (BF) for single units shifted from low (70 Hz) in the winter to high (140 Hz) in the summer. No difference was detected in auditory threshold at BF. Plasma testosterone (T) and estradiol (E2) levels in females are highest during the breeding season, suggesting that reproductive hormones may modulate this seasonal shift in auditory processing (Sisneros, Forlano, Knapp et al., 2004). Sisneros et al. (2004) demonstrated that the increase in phase-locking precision at BF for higher frequencies is in fact mediated by circulating T and E2. This frequency-dependent shift in peripheral encoding presumably increases detection of the higher harmonic components of the male mating hum during the summer.

Sex differences in seasonal variation and in auditory processing within the breeding season have not been addressed in this system. Despite the simple nature of the advertisement hum, males and females do need to process more complex stimuli due to

overlapping hums from multiple males and temporally modulated aggressive calls used in male-male interactions. Neural mechanisms for processing these more complex stimuli have been demonstrated in the auditory midbrain, the torus semicircularis (TS) (reviewed in Bass et al., 2005). Auditory processing of complex stimuli in the TS may exhibit sex differences due to the sexual dimorphism in the social contexts in which these stimuli are processed although this hypothesis has yet to be tested.

SONGBIRDS

Investigations on the role of sex differences in auditory processing in birds have focused on songbirds. Songbirds have been used as a model system for both vocal learning and behavioral neuroendocrinology. There are no clear examples of a sex difference in auditory processing, outside the song learning nuclei, which results in a difference in behavioral response to a species-typical vocalization. Sex differences in behavioral discrimination of variation in communication signals have been demonstrated, with females usually displaying more discrimination (Beletsky et al., 1980; Brenowitz, 1982a, 1982b; Nowicki et al., 2001; Searcy, Balaban et al., 1981; Searcy & Brenowitz, 1988; Searcy, Marler et al., 1981; although see Vicario et al., 2001 for an exception).

The focus on neural processing in songbird vocal learning has resulted in a greater understanding about differences at higher order auditory processing centers. One telencephalic nucleus, the caudal mesopallium (CM), is analogous to mammalian secondary auditory cortex and is not directly part of the song learning system. The caudal lateral mesopallium (CLM) shows evidence of sex differences in the processing of

conspecific signals in the zebra finch (*Taeniopygia guttata*) (Grace et al., 2003; Hauber et al., 2007). CLM neurons in males selectively respond to conspecific song over simple stimuli such as tones and white noise. CLM neurons in females do not show such selectivity. Presumably, this is due to a stimulus-dependent sex difference but since the sex comparison is between two different studies, this remains unclear.

Interestingly, male zebra finches do show greater discriminatory behavior than females but this has not been demonstrated in response to male song. Both male and female zebra finches produce communication calls, named long calls, and a sex comparison suggests that males and females discriminate based on different components of the long call (Vicario et al., 2001). Males and females both respond to long calls with their own long call. Males respond more strongly to female calls than to male calls and behavior varies based on the fundamental frequency, stimulus duration and frequency modulation at the beginning of the stimulus call. Females also respond more strongly to other female calls but behavior varies based only on stimulus duration. A sex comparison of neural selectivity in CLM in response to the long call may provide insight to the neural mechanisms of discrimination behavior in response to conspecific vocalizations.

Neurophysiological investigations also are needed in species for which clear sex differences in behavioral discrimination of male song are known. For example, female red-wing blackbirds discriminate between a song produced by a conspecific male and a mimic of the conspecific male song made by a mocking bird (Searcy & Brenowitz, 1988). Males show no discrimination in response to the same stimuli (Brenowitz,

1982a). A sex comparison of auditory processing in CM of this species may shed light on the neural mechanisms of discrimination behavior in songbirds.

One study in a songbird suggests that steroid hormones influence auditory processing in a stimulus-dependent manner. Female songbirds show condition-dependent responses to male song depending on reproductive state. Female white-throated sparrows, *Zonotrichia albicollis*, perform copulation solicitation displays (CSD) in response to conspecific song only during the breeding season. Maney et al. (2006) demonstrated that females treated with E2 respond with CSD to conspecific song but not to spectrally-matched tones. Control animals did not respond to any stimuli. The neural response to songs and tones was then determined by measuring the expression of the immediate early gene *zenk*. *Zenk* was measured in two forebrain analogues of mammalian auditory cortex, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM). In addition, *zenk* was measured in the avian homologue of the mammalian inferior colliculus, the nucleus mesencephalicus lateralis, pars dorsalis (MLd). E2 treatment resulted in significantly greater *zenk* expression in response to song when compared to the response to tones in all three auditory nuclei. In the forebrain nuclei, this was due to a selective decrease in the response to tones demonstrating a stimulus-dependent effect.

Whether or not this E2 related increase in selectivity is the result of estradiol acting directly on the auditory nuclei or on the response of afferent connections from earlier auditory nuclei, remains unknown. Considering that auditory processing in midbrain and forebrain nuclei relies on sensory information from lower order nuclei and the auditory periphery, hormone influences may occur in more primary auditory

processing. Two seasonal studies of auditory brainstem responses in a range of songbird species support this hypothesis with seasonal differences occurring in response to both clicks and tones (Lucas et al., 2002; Lucas et al., 2007). Whether or not reproductive hormones are involved in these seasonal changes is unknown. Furthermore, the link between seasonal differences and the processing of vocal signals remains to be addressed. A greater understanding of how reproductive hormones modulate the encoding of auditory information in songbirds would strengthen the understanding of the role of hormones in vocal communication and learning in birds and other taxa.

HUMANS

Human psychoacoustics and auditory physiology have a long history of demonstrating sex differences in auditory processing beginning in the late 1930's and continuing today (reviewed in McFadden, 1998). Additionally, the relationship between female reproductive condition and auditory processing has received a great deal of attention. Much of what is known pertains to the processing of synthetic stimuli such as tones, clicks, and noise bursts. How this directly relates to sex differences and hormone influences in the perception of voice remains unknown.

One study addressed sex differences in voice perception based on spectral characteristics (Hunter et al., 2005). Subjects were presented with a male voice that was either high-pass filtered or low-pass filtered. In a two alternative forced-choice test they were asked to compare an unfiltered voice with one of the filtered voices and decide which one sounded "real". Females were significantly more sensitive to the removal of the low frequency components of the voice than males were. Males and females did not

differ in sensitivity to the removal of the high frequency components of the voice. This result suggests that sex differences in voice perception are limited to a particular spectral band of the male voice. The authors of this study point out that it remains unknown whether this effect is specific to perception of the male voice (stimulus-specific) or applies to perception of all human voice.

Testing the hypothesis that sex differences are stimulus-dependent requires a careful selection of experimental paradigms and test stimuli. A study has attempted to address the neural mechanisms of sex differences in auditory discrimination of natural sounds. This study reports a sex difference in neural activation while performing an auditory recognition task but no difference in behavior (Maeder et al., 2001). The recognition task in this study used natural auditory scenes but none of the stimuli were human voices. Considering the sexual dimorphism in spectral characteristics of voice and the importance of voice in social communication, we might expect sex differences to be largest in response to auditory stimuli that convey information about the speaker's sex as discussed above (Hunter et al., 2005). Experiments that focus on neural activation during tasks that are known to exhibit sex differences may be more likely to detect differences in the processing of these signals.

One study has investigated sex differences in neural activation during voice recognition although fluctuations in female auditory processing may confound the results. While being monitored under fMRI, subjects were presented with stimuli from four categories: 1) male voice, 2) male voice with the fundamental frequency shifted up in the female direction, 3) female voice and 4) female voice with the fundamental frequency shifted down in the male direction. The subjects were presented one stimulus at a time and asked to indicate whether the voice was natural or unnatural. This test resulted in no sex differences in either behavior or cerebral blood flow during the task. The

reproductive state of females in this study is unknown. Another potential problem in studies of sex differences is that females are known to cycle in auditory processing along with the ovulatory cycle. In fact, during the preovulatory period, females are more male-like on several measurements of hearing (reviewed in McFadden, 1998). Sex differences may be dependent on the reproductive condition of females. Such results would also suggest, at least in females, that circulating reproductive hormones influence the function of the auditory system in adults.

The ovulatiory-shift hypothesis for socially monogamous species suggests that female preferences for male vocal characteristics will be condition-dependent based on reproductive state (Gangestad et al., 2005). Two studies have addressed this prediction in humans. Puts (2005) presented female listeners with male voices that had a raised vocal pitch, lowered vocal pitch or were unmodified. Females were asked to rate the voices on attractiveness for short-term and long-term relationships. Females in the most fertile phase of the menstrual cycle rated voices with a lower vocal pitch as more attractive for short term-term relationships. Feinberg et al. (2006) demonstrated similar results but did not consider the social context of preferences. Results from this study do suggest that preferences may not be related to shifts in peripheral sensory processing because females did not become more sensitive to the degree of masculinity among voices. Whether variation in preference is the result of changes in the encoding of the vocal signal remain unresolved. The corresponding variation that occurs in peripheral auditory processing in response to simple stimuli (reviewed in McFadden, 1998) suggests at least a partial role for peripheral changes in the perception of human voice. Investigations into sub-cortical neural responses across the ovulatory cycle to variations in masculinity may help clarify these issues.

AMPHIBIANS

This dissertation focuses on anuran amphibians. Anurans have been the subject of numerous behavioral and neuroethological studies of animal communication and auditory processing (Gerhardt & Bee, 2006; Rose & Gooler, 2006). Sex differences and hormone influences also have been examined at multiple levels of the communication system (Wilczynski et al., 2005).

Anuran amphibians exhibit sexual dimorphisms in behavioral responses to the male advertisement call. Sexes differ in responses to different spectral components of the call (Narins & Capranica, 1976), recognition of calls as conspecific (Bernal et al., 2007) and discrimination between stimuli to which they will respond (Schwartz, 1987; Schwartz & Wells, 1985). All of these types of sex differences could result from differences in how the sensory system encodes the auditory stimulus.

The clearest example of sex differences in behavior relating to differences in neural processing of a communication signal have been demonstrated in the coqui, *Eleutherodactylus coqui* (Narins & Capranica, 1976). Males produce an advertisement call with two temporally and spectrally different components. The "co" note stimulates the lower spectral band of the hearing range in this species and has no frequency modulation. The "qui" component is an upward frequency sweep that stimulates the upper spectral band of the hearing range. The "co" note alone evokes a strong territorial calling response in males whereas the "qui" alone does not. The opposite is true for the female phonotactic response with females attracted to the "qui" alone but not the "co" note alone. Corresponding sex differences are found in the peripheral auditory system. Eighth nerve units can be classified into low-, mid- and high-frequency based on their

best excitatory frequency (BF). Mean BF for "low" units in males are significantly higher and more closely tuned to the "co" note than in females. Mean BF for "high" units are lower in females and more closely tuned to the "qui" component of the call than in males. These results suggest that sex differences in the spectral selectivity of the peripheral auditory system result in behavioral differences that are frequency-dependent.

Other sex differences in frequency selectivity have been demonstrated in the peripheral auditory system in several anuran species (Keddy-Hector et al., 1992; McClelland et al., 1997; Vassilakis et al., 2004; Wilczynski et al., 1992; Wilczynski et al., 1984). Some of these sex differences are associated with strong correlations in size dimorphisms in several morphological characteristics. The relationship between auditory tuning and peripheral morphology suggest that at least some of the sex differences in behavior and auditory processing are due to developmental differences.

Some evidence suggests that auditory processing may also be influenced by circulating steroid hormones in adults. In the male green treefrog, *Hyla cinerea*, midbrain auditory neural thresholds were significantly lower at three of 24 frequencies in gonadectomized males compared to intact males (Penna et al., 1992). Whether or not these shifts in sensitivity influence the processing of conspecific communication signals is unknown. Additionally, injection of E2 in the third ventricle of the female leopard frog, *Rana pipiens*, increased the amplitude of midbrain auditory evoked potentials in response to all of four different pure-tone stimuli (Yovanof & Feng, 1983). The four tones were chosen to represent the frequencies contained in the male advertisement call. It remains unclear whether estradiol induces a general elevation in the response of the auditory system or a frequency-dependent elevation limited to the spectral band of the

advertisement call. This distinction is important because a selective effect on the processing of communication signals would suggest an enhancement in the filtering properties of the auditory system.

The clearest example of hormone influences on behavior through modulation of auditory processing has been demonstrated in the túngara frog, *Physalaemus pustulosus*. Females demonstrate condition-dependent mating behavior in relation to time to ovipisition (Lynch et al., 2005). This behavioral variation is regulated by gonadal steroids (Lynch et al., 2006; Lynch & Wilczynski, 2005). Neural activation, measured by the expression of the immediate early gene *zenk*, revealed that reproductive hormones significantly increase *zenk* expression in the auditory midbrain in response to conspecific vocalizations (Lynch & Wilczynski, in press). These results demonstrate that reproductive hormones increase the response of the auditory system to communication signals. Whether or not the influence is frequency-dependent or stimulus-dependent, remains to be explored.

Questions remain about the neural mechanisms that result in sex- and hormone-related differences in behavioral responses to auditory signals. Much of the evidence for influences on auditory processing is based on the quietest sounds that the system will respond to. BF and frequency selectivity at threshold often differ significantly when compared to responses to stimuli above threshold (Capranica, 1992; Schwartz & Gerhardt, 1998). Considering that frogs commonly produce signals at levels well above the threshold for detecting them, an understanding of sex differences and hormone influences in auditory processing requires assessment at these higher stimulus levels in addition to threshold levels. Also, most studies have described differences in auditory

processing in response to pure tones but not auditory communication signals. The response of a neuron is often not linear across both simple and complex stimuli (Eggermont et al., 1983; Rose & Capranica, 1983; Rose & Capranica, 1985; Theunissen et al., 2000; Woolley et al., 2006), suggesting that sex differences may vary depending on the stimulus. Studies including both simple and natural stimuli may help clarify the role of reproductive hormones in the neural processing of communication signals.

The auditory midbrain

In order to address the questions presented above, this dissertation examines neural responses in the torus semicircularis (TS), the amphibian midbrain homolog of the inferior colliculus. The TS is a likely area for reproductive hormones to influence the processing of vocal signals. First, the TS integrates the vast majority of ascending auditory inputs between the brainstem auditory nuclei and the forebrain (Wilczynski & Endepols, 2007). This integration results in a specialization within the TS for processing complex stimuli, such as communication signals, in addition to the more simple pure-tone stimuli (Feng & Ratnam, 2000; Rose & Gooler, 2006). Second, auditory processing in the TS is important in auditory detection and phonotactic behavior through its projections to forebrain nuclei and brainstem motor areas (Wilczynski & Endepols, 2007). Behavioral audiograms match closely to neural audiograms recorded from the TS in several anuran species including *Hyla cinerea* (Bibikov & Elepfandt, 2005; Elepfandt et al., 2000; Megela-Simmons et al., 1985). Lesions of the TS in *Hyla versicolor* also eliminate phonotactic behavior in two-alternative choice tests while extensive thalamic lesions do not (Endepols et al., 2003). Last, the TS is sensitive to steroid hormones with

evidence for receptors reported in *Xenopus laevis* (Kelley, 1980) and *Rana esculenta* (Dimeglio et al., 1987; Guerriero et al., 2005).

CONCLUSIONS

By considering what is known about sex differences in auditory evoked behavior and physiology across vertebrate taxa, some principles emerge. Sex differences in behavior have primarily been demonstrated in discrimination tasks. Discrimination among vocal signals can be based on a variety of signal parameters including spectral bands, fundamental frequency and temporal characteristics. Studies in frogs and humans tell us that sex differences may be limited to particular spectral bands. Additionally, these studies suggest that the female auditory system fluctuates in the way it processes vocal signals. The appearance of a sex difference may depend on the reproductive condition of the female. When possible, future studies should compare male auditory processing to that of females in different reproductive conditions. Alternatively, some sex differences in behavior may not have an underlying sensory mechanism in some social systems. Most of the examples of sex differences in behavior are based on comparisons where males and females perform different tasks. For example, in frogs, males call in response to the call of another male and females approach the calling male. In at least one example, an established sex difference in species recognition for *P. pustulosus* (Bernal et al., 2007) is task specific (Bernal, 2007). When both sexes perform a phonotaxis response, they exhibit no differences.

Principles also emerge when looking at the role of reproductive hormones in modulating auditory processing in females. The influence of hormones can be specific to a particular spectral band (frequency-specific) and can be limited to a particular stimulus type (stimulus-specific). Studies in fish demonstrate that non-reproductive females have a frequency-specific reduction in the ability to encode the upper harmonics of the male advertisement signal. E2 administration restores that encoding ability. A study in songbirds demonstrates that E2 increases selectivity of the neural response to male song in a stimulus-specific manner by reducing responsiveness to pure tones. The reduced response to pure tones coincides with an increased reproductive responsiveness to male song. When feasible, future studies on the role of reproductive hormones in modulating auditory processing should include considerations of both spectral characteristics and stimulus type.

The research presented in this dissertation incorporates some of the recommendations of the above review in studies of auditory processing in the green treefrog, *Hyla cinerea*. I test the hypothesis that reproductive hormones influence the auditory processing of communication signals in a frequency- and stimulus-dependent manner. I present electrophysiology experiments from the auditory midbrain that assess both auditory sensitivity and neural responses to stimuli consistent with close-range communication. The experiments in chapter two compare multiunit auditory sensitivities between males and females in response to pure tones and the male advertisement call. Chapter two also tests the prediction that circulating androgens modulate auditory processing for these same stimuli. The experiments in chapter three test the prediction that sex differences depend on the reproductive state of the female. Neural responses in

unmated and postmated females are compared to male responses. The work in this chapter also tests the prediction that differences will be frequency- and stimulus-dependent by using both natural advertisement call and white noise stimuli. Experiments in chapter four attempt to address the mechanisms of the variation in auditory processing demonstrated in the previous chapters. The work in this chapter specifically tests whether modulation of the hypothalamic-pituitary-gonadal axis, which includes activation of luteinizing hormone receptors and steroid manipulation, influences auditory processing of communication signals. Again, these experiments predict that hormone influences will be frequency- and stimulus-dependent.

Chapter 2: Sex differences and androgen influences on midbrain auditory thresholds in the green treefrog, *Hyla cinerea*

INTRODUCTION

Reproductive hormones are well established modulators of communication systems in fish (Stoddard et al., 2006), amphibians (Wilczynski et al., 2005) and birds (Ball et al., 2002) with effects readily observable at the level of behavior. Steroid hormones are known to act at forebrain nuclei to influence the motivation to communicate or respond to signals. These forebrain nuclei rely on input from lower sensory processing areas and evidence suggests that hormones act on the sensory system to modulate processing of communication stimuli (Maney et al., 2006; Sisneros et al., 2004; Zakon, 1987).

Sex differences in behavior suggest that reproductive hormones play a role in sensory processing, either during early development or the adult life of the animal. In anuran amphibians, males produce vocal signals that communicate with both females and other males. The same vocalization will evoke different behavioral responses in each sex, which may be due in part to differences in early auditory processing. Sex differences in anurans have been demonstrated in peripheral auditory processing of simple, pure-tone stimuli (Keddy-Hector et al., 1992; McClelland et al., 1997; Narins and

Capranica, 1976; Vassilakis et al., 2004; Wilczynski et al., 1992; Wilczynski et al., 1984). Considering the behavioral importance of complex communication signals (Gerhardt and Bee, 2006), and the processing of these stimuli in the central nervous system, we investigated sex differences in the processing of both simple and complex stimuli in the central nervous system of adult anurans. Additionally, we tested whether the neural responses to these stimuli were modulated by circulating androgens.

The auditory midbrain, the torus semicircularis (TS), is an excellent starting point for investigating the role of reproductive hormones in modulating auditory processing of communication signals. The TS integrates the vast majority of ascending auditory inputs between the brainstem auditory nuclei and the forebrain (Wilczynski and Capranica, 1984; Wilczynski and Endepols, 2006). This integration results in a specialization within the TS for processing complex stimuli, such as communication signals, in addition to more simple pure-tone stimuli (Feng and Ratnam, 2000; Rose and Gooler, 2006). The TS is also sensitive to testosterone with evidence for receptors reported in *Xenopus laevis* (Kelley, 1980) and *Rana esculenta* (Dimeglio et al., 1987; Guerriero et al., 2005). Single-unit and multiunit neural responses in the TS vary seasonally (Goense and Feng, 2005; Hillery, 1984; Walkowiak, 1980) suggesting a role for steroid hormones in modulating these responses. Additionally, gonad removal influences the multiunit audiogram in the TS of male *Hyla cinerea* (Penna et al., 1992). The relevance of hormonal influence in the TS to the processing of communication signals is likely considerable although unknown.

Sex and testosterone may influence auditory sensitivity at all frequencies within the hearing range of the animal or only at the spectral sensitivity peaks. The inner ear of

the anuran contains two physically and functionally different sensory end-organs that process different spectral bands of airborne auditory stimuli (Smotherman and Narins, 2000). The amphibian papilla (AP) is a tonotopic structure that responds to a spectral band of approximately 100-1400 Hz for *H. cinerea*. The AP has two sensitivity peaks, the low best frequency (LBF) and the mid best frequency (MBF). The basilar papilla (BP) is an end-organ that responds at a single best excitatory frequency, the high best frequency (HBF), and processes frequencies in the higher range, between 1600-5000 Hz. Both the AP and BP spectral bands are represented in the TS, with multiunit audiograms showing a LBF, MBF and HBF. At the level of the TS, *H. cinerea* is more sensitive at the LBF and MBF of the AP compared to the HBF of the BP (Lombard and Straughan, 1974), however, sex and testosterone may influence this relationship. Additionally, evidence suggests sexes may differ in the frequency at which they are most sensitive, particularly at the HBF (Keddy-Hector et al., 1992; McClelland et al., 1997; Narins and Capranica, 1976; Wilczynski et al., 1984).

Sex and testosterone also may influence the spectral filtering properties of the auditory system. The matched filter hypothesis (Capranica and Moffat, 1983) suggests that dominant frequencies in the male advertisement call match the peak sensitivities of the auditory system. Gerhardt (1974) reported the natural range of dominant frequencies in the male advertisement call for a population of *H. cinerea* and the MBF and HBF peaks of the neural audiogram both fall within that range. The influences of sex and testosterone may be limited to frequencies inside or outside the advertisement call range, suggesting an influence on the auditory filter.

The neural audiogram has long been used to relate neural sensitivity in response to pure tones with behavioral responses to more complex communication signals. Some evidence suggests that, at the single unit level, neural processing of complex stimuli is not simply the linear summation of a neuron's response to pure tones (Rose and Capranica, 1983; Rose and Capranica, 1985; Woolley et al., 2006). These stimulus-specific responses suggest that multiunit thresholds also may depend on the type of stimulus and that sex and testosterone may influence thresholds in a stimulus-specific manner.

In this study we tested the hypothesis that sex differences influence neural sensitivity to auditory stimuli in a frequency-dependent manner based on peak sensitivities in the auditory system and on spectral characteristics found in the male advertisement call. We also tested the hypothesis that sex differences will be stimulus-dependent by examining thresholds in response to pure tones and the male advertisement call. Lastly we tested whether males and females differ in the modulation of these responses by testosterone.

MATERIALS AND METHODS

Animal Care

We purchased adult male and female green treefrogs (*Hyla cinerea)* from two suppliers, NASCO (Fort Atkinson, WI) and Charles Sullivan Co. (Nashville, TN), and housed them in small groups of six animals per 10 gallon aquarium for at least two weeks to acclimate to lab conditions. We fed the frogs crickets *ad libitum* and provided water in

a bowl inside each aquarium. Environmental conditions were 23ºC and 14:10 light:dark cycle. All procedures were performed in accordance with a protocol approved by The University of Texas at Austin Institutional Animal Care and Use Committee.

Hormone manipulation

For the surgical implantation of testosterone, we anesthetized animals by immersion in 2.5% urethane and made a small dorsal cutaneous incision. All individuals were left gonadaly intact and all received subcutaneous implants with Silastic© capsules (1.47mm i.d. x 1.96mm o.d. x 7mm total length) filled with either testosterone (male: n=7, female: n=3) or cholesterol (control, male: n=7, female: n=7) (Burmeister and Wilczynski, 2001). We sealed the incision with Vetbond (World Precision Instruments, Sarasota, FL) and placed each animal in its own holding aquarium for 10-12 days. Following the collection of electrophysiological data, we immediately euthanized the animals and collected a blood sample for hormone analysis. To verify the effectiveness of the testosterone manipulation, we used an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). General methods for the EIA procedure have been previously described (Lynch and Wilczynski, 2006). For some animals, we did not collect a sufficient amount of plasma or we could not reliably assay plasma hormone levels. Those animals were omitted from the hormone analysis. The measured androgen levels were the following (mean ± SEM): a) control males 4.7 ± 1.1 ng/ml (n=6), b) control females 6.9 ± 1.2 ng/ml (n=7), c) testosterone-treated males 162.3 ±45.5 ng/ml (n=3) and d) testosterone-treated females 257.1 ±92.3 ng/ml (n=3).

Neurophysiology preparation

Neurophysiological methods were described previously by Wilczynski et al. (1993). Eight to ten days after the testosterone implant, we anesthetized animals in a solution of 2.5% tricaine methanesulfonate (Sigma, St. Louis, MO) and cut a section of skin that we folded back to expose the braincase covering the midbrain. To expose the midbrain we cut away a small piece of braincase and replaced it with a damp piece of tissue. We replaced the skin over the exposed area and allowed the animal to recover for two days in its own holding aquarium. On the day of the electrophysiological recordings, we immobilized the animal with an intramuscular injection of curare (d-tubocurarine chloride; 10 μg/g body weight) and applied 2% lidocaine as a local anesthetic to the tissue surrounding the exposed brain area. Recordings took place in an Industrial Acoustics sound-attenuating chamber with the animal draped in wet paper towels and at an ambient temperature of 23°C.

Neurophysiology and auditory stimuli

Procedures are detailed in previous studies (McClelland et al., 1997; Wilczynski et al., 1993). Sound was presented to the ear of the animal through a custom made, closed-field earphone system. We used a Brüel & Kjær Digital Precision Integrating Sound Level Meter Type 2230 to calibrate the earphone system and determine the stimulus amplitude at the animal's ear. Tone production and attenuation were controlled by custom designed hardware. Single frequency tone stimuli (300 ms duration repeated every 1.6 s) at 100-1000 Hz (in 100 Hz steps) and 1200-5000 Hz (in 200 Hz steps) were used to determine midbrain auditory thresholds within the hearing range of this species.

We assessed acoustically-evoked extracellular multiunit responses in the TS contralateral to the earphone, using a low impedance (0.5-1.5 MΩ) tungsten electrode (A-M Systems, Sequim, WA). Along with the recording electrode, we placed a microinjection needle in the tectal ventricle above the TS on the side opposite the recording site for another study for which the data will not be presented. We identified the TS by its robust response to a search stimulus consisting of two tones (900 Hz and 3000 Hz) presented simultaneously at a peak amplitude of 80 dB SPL. To establish audiograms, we determined auditory thresholds at each frequency by adjusting the attenuation level of the stimulus in 10 dB and then in 1 dB SPL steps, stopping at the lowest sound pressure level that evoked a reliable response as monitored by the observer visually on a storage oscilloscope and acoustically through earphones.

We also determined auditory thresholds to a field-recorded advertisement call from a single male *H. cinerea* in Travis County, TX. The advertisement call was 122 ms in duration with spectral peaks at 825 and 2668 Hz. The characteristics of this call fell within the range of variation described previously (Gerhardt, 1968, 1974).

Statistical analysis

For measures of audiogram best frequency, we identified a LBF, MBF and HBF for each animal. Using two-way ANOVA we tested whether sex or testosterone influenced the best frequency and the threshold at each of these peaks. To test the qualitative observation that the auditory system is more sensitive at the LBF and MBF than it is at the HBF (Lombard and Straughan, 1974), we compared the relative thresholds at these peaks. We calculated, for each subject, a ratio of the threshold at the

LBF to the threshold at the HBF (LBF/HBF) and a ratio of the threshold at the MBF and HBF (MBF/HBF). These ratios were then compared to a null hypothesis of 1.0 using a one-sample t-test. We also compared ratios among the groups using two-way ANOVA.

Comparisons of the audiograms between groups were analyzed using two-way and one-way multivariate analysis of variance (MANOVA). These analyses were conducted based on the reported range of spectral components in the male advertisement call as established by Gerhardt (1974). The male advertisement call contains two spectral peaks and the variation in the frequency components of those peaks had a range of 761-1340 Hz for the lower range and 2520-3600 Hz for the higher range. MANOVA was performed on thresholds that were grouped by whether or not their corresponding dependent variable (frequency of stimulus) fell within the natural bands of frequencies found at the peaks of the male advertisement call. Stimulus frequencies considered within the advertisement call bands were: 800, 900, 1000, 1200, 1400, 2600, 2800, 3000, 3200, 3400, and 3600 Hz (Figure 1a, vertical grey bars). Frequencies considered outside the bands were: 100, 200, 300, 400, 500, 600, 700, 1600, 1800, 2000, 2200, 2400, 3800, 4000, 4200, 4400, 4600, 4800, and 5000 Hz.

Comparisons of mean thresholds in response to the field-recorded male advertisement call were made using two-way ANOVA. A p-value for statistical significance was set at $p < 0.05$ for all tests in this study.

RESULTS

Neural Audiograms

Neural audiograms were typical of those previously reported for *Hyla cinerea* (Lombard and Straughan, 1974; Penna et al., 1992) (Figure 1a). When males and females in the control condition were considered together, the lower frequency range (900-1400 Hz), corresponding to the amphibian papilla (AP), contained two sensitivity peaks. The first peak, the low best frequency (LBF), had a mean of 446 Hz with a between subject range of 300-600 Hz. The second peak, the mid best frequency (MBF), had a mean of 907 Hz with a between-subject range of 800-1000 Hz. The higher frequency range (1600-5000 Hz), corresponding to the basilar papilla (BP), contained a single peak, the high best frequency (HBF), with a mean of 2343 Hz and a range between 1900-2800 Hz.

Sex comparisons in the control condition

Audiogram best frequencies

Males and females did not differ in the frequencies at which they were most sensitive. The mean LBF for females was 493 ± 32 Hz and for males was 457 ± 23 Hz (Figure 2a) with no main effect of sex [$F(1, 20)= 0.570$, $p=0.459$]. The mean MBF for females was 971 ± 43 Hz and for males was 929 ± 34 Hz (Figure 2b) with a trend toward a main effect of sex [$F(1, 20)= 4.125$, $p= 0.056$]. A post-hoc analysis comparing males and females in the control condition revealed no evidence for a sex difference in the MBF [Bonferroni post-hoc test, $t(20)= 0.903$, $p= 0.377$]. The mean HBF for females was 2343 ± 84 Hz and for males was 2314 ± 116 Hz (Figure 2c) with no significant sex difference [Bonferroni post-hoc test, $t(20)= 0.207$, $p= 0.838$]. Previous studies in other anuran

species demonstrate that, for some species, size correlates negatively with VIIIth nerve best excitatory frequency (BEF) in the BP range (corresponding here to the HBF in the TS) and significantly influences sex differences (McClelland et al., 1997; Shofner and Feng, 1981). We found no such correlation in *H. cinerea* at the level of the TS. For the animals in this study, male snout-vent length (SVL) was significantly greater than in females [male: 50.4 mm ± 0.6 N=7, female 46.3 mm ± 1.0, $t(12)=3.568$, p=0.004] which is consistent with previous reports (Garton and Brandon, 1975). SVL did not correlate with HBF [r (24) = -0.131, p = 0.542] and therefore did not influence the sex comparison.

Threshold at audiogram best frequencies

Males had lower thresholds than females at LBF and MBF but not HBF. The mean threshold at LBF for females was 44.9 ± 0.6 dB SPL compared to 38.6 ± 1.2 dB SPL for males (Figure 3a, control) with a significant main effect of sex [F(1, 20)= 30.24, p<0.0001]. The mean threshold at MBF for females was 48.9 ± 0.8 dB SPL and for males was 44.3 ± 1.6 dB SPL (Figure 3b, control) with a significant main effect of sex [F(1, 20)= 10.32, p= 0.004]. The mean threshold at HBF for females was 46.0 ± 1.0 dB SPL compared to 46.7 ± 2.1 dB SPL for males (Figure 3c, control) with no main effect of sex [F(1, 20)= 1.158, p= 0.295].

Relative sensitivities of audiogram peaks

Sex differences in threshold at LBF resulted in males showing a greater sensitivity at LBF compared to HBF and females showing equal sensitivity between the two peaks. The mean LBF/HBF threshold ratio for males was 0.83 ± 0.02 (Figure 4a, control) which

was significantly lower than one [one sample t-test, $t(6)= 9.181$, $p < 0.0001$]. Threshold at LBF in males was lower by 8.1 ± 1.2 dB SPL compared to threshold at HBF. The mean LBF/HBF threshold ratio in females was 0.98 ± 0.03 (Figure 4a, control) which was not significantly different from 1.0 [one sample t-test, $t(6)= 0.6563$, $p= 0.536$]. Directly comparing the LBF/HBF threshold ratio between males and females demonstrated that males had significantly lower ratios than females [Bonferroni post-hoc test, $t(20)= 4.540$, $p = 0.0002$].

Sex differences in threshold at MBF resulted in males showing a greater sensitivity at MBF compared to HBF and females showing less sensitivity at MBF compared to HBF. The mean MBF/HBF threshold ratio for males was 0.95 ± 0.02 with a trend toward a difference from a ratio of 1.0 [one sample t-test, $t(6)= 2.315$, $p= 0.060$] (Figure 4b, control). The threshold at MBF in males was lower by 2.4 ± 1.1 dB SPL compared to threshold at HBF. The mean MBF/HBF threshold ratio in females was 1.06 ± 0.02 which was significantly higher than 1.0 [one sample t-test, $t(6)= 2.919$, $p= 0.027$] (Figure 4b, control). The threshold at MBF in females was higher by 2.9 ± 1.0 dB SPL compared to threshold at HBF. Directly comparing the MBF/HBF ratio between males and females demonstrated that males had significantly lower ratios than females [Bonferroni post-hoc test, $t(20)= 3.179$, $p = 0.005$].

Audiogram frequencies and the male advertisement call spectral bands

Females had significantly higher thresholds than males at frequencies outside the bands of the advertisement call [one-way MANOVA, $F(1,12)=6.94$, $p=0.022$] (Figure 5b, control). Visual inspection of the audiograms shows that females tended to have higher

thresholds at the upper and lower ends of the curve but not at the intermediate frequencies (Figure 1b). In contrast, thresholds in response to frequencies within the spectral bands of the male advertisement call were not significantly different between the sexes [one-way MANOVA, $F(1,12)=0.13$, $p=0.720$] (Figure 5a, control).

Thresholds to male advertisement call

In response to the male advertisement call stimulus, females had significantly lower thresholds than males [Bonferroni post-hoc test, $t(12)= 3.706$, $p= 0.003$]. Mean threshold for females was 52.1 ± 0.4 dB SPL compared to 57.4 ± 1.2 dB SPL for males (Figure 6, control).

Testosterone treatment

Audiogram best frequencies

Testosterone had no significant influence on audiogram LBF in either females or males, with no interaction effect [$F(1,20)=0.0029$, $p=0.958$] and no overall treatment effect [$F(1, 20)=0.2910$, $p=0.459$]. The mean LBF for females treated with testosterone was 467 ± 120 Hz compared to 493 ± 32 Hz for control females (Figure 7a). The mean LBF for males treated with testosterone was 436 ± 36 Hz compared to 457 ± 23 Hz in control males (Figure 7d).

Testosterone did not significantly influence MBF in either females or males, with no interaction effect [$F(1,20)=0.8523$, $p=0.367$] and no overall treatment effect [$F(1, 20)= 0.0341$, $p= 0.855$]. The mean MBF for females treated with testosterone was 1000 ± 00

Hz compared to 971 ± 44 Hz for control females (Figure 7b). The mean MBF for males treated with testosterone was 886 ± 26 Hz compared to 929 ± 34 Hz in control males (Figure 7e).

Testosterone did influence HBF in females but not males, with a trend toward an interaction effect [$F(1, 20)= 3.043$, $p= 0.096$]. Females treated with testosterone showed a trend toward a lower HBF with a mean of 2000 ± 200 Hz compared to 2343 ± 84 Hz for control females [Bonferroni post-hoc test, $t(20)=1.891$, $p = 0.073$] (Figure 7c). Males showed no significant influence of testosterone treatment with a mean HBF of 2371 ± 81 Hz for males treated with testosterone compared to 2314 ± 116 Hz in control males [Bonferroni post-hoc test, $t(20)=0.4070$, $p = 0.688$] (Figure 7f).

Threshold at audiogram best frequencies

Testosterone did not significantly influence threshold at LBF in either females or males, with no interaction effect [$F(1,20)=0.1432$, $p=0.709$] and no overall treatment effect [$F(1, 20)= 0.6785$, $p = 0.420$]. The mean threshold at LBF for testosterone-treated females was 45.3 ± 0.9 dB SPL compared to 44.9 ± 0.6 dB SPL for control females (Figure 3a). The mean threshold at LBF for testosterone-treated males was 39.9 ± 1.0 dB SPL compared to 38.6 ± 1.2 dB SPL for control males (Figure 3a).

Testosterone did not significantly influence threshold at MBF in either females or males, with no interaction effect [$F(1,20)=0.0887$, $p=0.769$] and no overall treatment effect [$F(1, 20) = 0.9574$, $p = 0.340$]. The mean threshold at MBF for testosterone-treated females was 51.0 ± 2.0 dB SPL compared to control females with 48.9 ± 0.8 dB

SPL (Figure 3b). The mean threshold at MBF for testosterone-treated males was 45.4 ± 1.8 dB SPL compared to control males with 44.3 ± 1.6 dB SPL (Figure 3b).

Testosterone did not significantly influence threshold at HBF in either females or males with no interaction effect [F(1,20)=2.146, p=0.158] and no overall treatment effect [F(1, 20) = 0.2826, p= 0.601]. The mean threshold at HBF for testosterone-treated females was 49.7 ± 0.7 dB SPL and for control females was 46.0 ± 1.0 dB SPL (Figure 3c). The mean threshold at HBF for testosterone-treated males was 45.0 ± 1.7 dB SPL and for control males was 46.7 ± 2.1 dB SPL (Figure 3c).

Relative sensitivities of audiogram peaks

Testosterone influenced the LBF/HBF threshold ratio in a sex specific manner as supported by a significant interaction effect between sex and testosterone [F(1,20)=5.266, p=0.033] (Figure 4a). The interaction effect was due to a small, non-significant decrease in the ratio in females and a small non-significant increase in the ratio in males. First, testosterone did not significantly influence the ratio in females. Testosterone-treated females had a mean LBF/HBF threshold ratio of 0.91 ± 0.03 compared to the control mean of 0.98 ± 0.03 [Bonferroni post-hoc test, $t(20)= 1.564$, p= 0.134]. Testosterone-treated females did have a mean ratio that showed a trend toward a difference from 1.0 [one sample t-test, $t(2)= 2.982$, p= 0.096]. The threshold at LBF in females treated with testosterone was lower by 4.3 ± 1.5 dB SPL compared to threshold at HBF. The mean LBF/HBF threshold ratio in males treated with testosterone was 0.89 ± 0.02, showing a trend toward an increase when compared to the control mean of 0.83 ± 0.02 [Bonferroni post test , $t(20)= 1.728$, p= 0.099]. Despite the increase, the mean LBF/HBF ratio in

testosterone-treated males remained significantly lower than 1.0 [one sample t-test, $t(6)= 5.460$, $p= 0.002$]. The threshold at LBF in males treated with testosterone was lower by 5.1 ± 1.1 dB SPL compared to threshold at HBF.

Testosterone did not influence the MBF/HBF threshold ratio in either males or females with no interaction effect [$F(1,20)=2.836$, $p=0.108$] and no overall treatment effect [$F(1,20)=0.1491$, $p=0.704$] (Figure 4b). Testosterone-treated females had a mean MBF/HBF threshold ratio of 1.03 ± 0.04 compared to the control mean of 1.06 ± 0.02. The mean MBF/HBF threshold ratio in males treated with testosterone was 1.01 ± 0.03 compared to the control mean of 0.95 ± 0.02.

Audiogram frequencies and the male advertisement call spectral bands

Testosterone treatment influenced audiograms in a sex and frequency dependent manner (Figure 8). For frequencies corresponding to the spectral bands of the male advertisement call, females treated with testosterone had significantly elevated thresholds compared to control females [one-way MANOVA, $F(1,8)=11.59$, $p=0.009$] (Figure 5a). Thresholds in males treated with testosterone were not significantly different from control males [one-way MANOVA, $F(1,12)=0.04$, $p=0.838$] (Figure 5a), with a trend toward a sex by testosterone interaction effect [$F(1,20)=3.55$, $p=0.074$]. Testosterone treatment did not influence thresholds for frequencies outside the bands of the advertisement call in either males or females, with no interaction effect [$F(1,20)=.25$, $p=0.621$] and no overall effect of testosterone [$F(1,20)=.002$, $p=0.964$] (Figure 5b).

Thresholds to male advertisement call

For thresholds in response to the male advertisement call we found no significant interaction between sex and testosterone treatment [$F(1,20)=2.044$, $p=0.168$] and no overall effect of testosterone [$F(1,20)=1.709$, $p=0.206$]. Females treated with testosterone had a mean threshold of 55.3 ± 1.2 dB SPL compared to the control mean of 52.1 ± 0.4 dB SPL. The mean threshold for males treated with testosterone was 57.3 ± 1.2 dB SPL compared to 57.4 ± 1.2 dB SPL for the control condition (Figure 6). Despite the lack of a significant sex by testosterone interaction effect, testosterone treatment did elevate female thresholds such that they were not significantly different from control males [Bonferroni post test, $t(8)= 1.139$, $p=0.288$].

DISCUSSION

Sex differences

Our first goal for this study was to determine whether male and female *H. cinerea* differed in neural sensitivity to auditory stimuli. We tested whether sex differences to pure tones were frequency-dependent based on both peak sensitivities in the auditory system and spectral characteristics of the male advertisement call. Our results demonstrate sex differences in the relative sensitivities of the spectral peaks and in the sensitivity to frequencies outside the spectral bands of the advertisement call. Sex differences are also stimulus-dependent such that differences in response to pure tones do not predict the differences to the natural advertisement call.

Previous studies reported sex differences in the BP BEF from single units in the VIIIth nerve in other species, but we see no evidence of a related difference in *H. cinerea* at the level of the TS. Considering the consistency in peak sensitivities between responses from the VIIIth nerve and the TS in *H. cinerea* (Lombard and Straughan, 1974), if sex differences occurred in the peripheral auditory system we would expect to see them represented in the responses from the TS. For other species examined, females have BP units tuned to a lower frequency than males and the difference is associated with a dimorphism in SVL, with females larger than males (Keddy-Hector et al., 1992; McClelland et al., 1997; Narins and Capranica, 1976; Wilczynski et al., 1984). In a comparative study between *Hyla ebraccata* and *Hyla microcephala*, McClelland et al. (1997) demonstrated a relationship between the magnitude of a body size sex dimorphism and the existence of a dimorphism in BP BEF. *H. microcephala* shows a small sex dimorphism in size and no difference in BP BEF. *H. ebraccata* shows a larger dimorphism in size, and females are tuned to lower frequencies than males. For comparison, *E. coqui* shows a BP BEF dimorphism (Narins and Capranica, 1976) and also has a relatively large size dimorphism (Woolbright, 1983). *Acris crepitans* and *Hyla crucifer* offer some exceptions in that the size dimorphism between males and females is small yet females still have a lower BP BEF (Keddy-Hector et al., 1992; Wilczynski et al., 1984; Woolbright, 1983). This suggests that size may not be the only factor driving sex differences. *H. cinerea* also may provide evidence that other selection pressures drive tuning in the BP range. As demonstrated in Garton and Brandon (1975) and in this study, *H. cinerea* has a relatively small size dimorphism comparable to that of *H. crucifer*, *H. microcephala* and *A. crepitans*. However, unlike those other species, males

are significantly larger than females. One interpretation of the discordance between size dimorphism and BP tuning differences is that BP tuning may be the result of a combination of selective pressures that shape communication systems and size dimorphisms.

Multiunit thresholds to pure tones at the audiogram sensitivity peaks demonstrate that males are more sensitive than females at the two peaks that fall within the AP range of frequencies (LBF and HBF) but not at the peak in the BP range (HBF). The differences in thresholds result in a sex difference in the relative sensitivities between the AP and BP range of frequencies. Lombard and Straughan (1974) qualitatively observed that multiunit thresholds are lower at the AP sensitivity peaks compared to those at the BP peak. Results from our study statistically support this observation only in males. Females are equally sensitive at LBF and HBF. Additionally, females are less sensitive at MBF compared to HBF.

Strong sex differences emerge when the multiunit audiogram is considered in terms of the prominent frequencies in the natural vocalization of the species. Females are less sensitive than males in response to frequencies that fall outside the spectral bands of the male advertisement call. For frequencies that fall within the bands of the male advertisement call no sex difference is evident. These results demonstrate that, in response to pure-tone stimuli, sex differences are limited to particular frequency bands that are unlikely to be involved in the processing of communication signals.

Sex differences in multiunit thresholds are also stimulus-dependent. In contrast to the lack of a sex difference in response to pure tones within the spectral bands of the advertisement call, a sex difference emerges in response to the naturally recorded

advertisement call such that females are more sensitive to the behaviorally relevant stimulus. Additionally, when there is a sex difference in response to tones, females are less sensitive than males, which is opposite to the result in response to the advertisement call. This may be the result of stimulus-dependent response properties of subsets of cells within the TS such as those exhibited in *Rana pipiens* (Rose and Capranica, 1983; Rose and Capranica, 1985). A single unit analysis could attempt to test whether the stimulus-dependent sex differences in this study are due to the response properties of individual cells or an emergent property of a network of cells within the TS.

Testosterone influences

Our second goal for this study was to determine whether circulating testosterone levels influence neural sensitivity to auditory stimuli in male and female *H. cinerea*. Our results demonstrate that testosterone treatment influences multiunit auditory thresholds in females but we find very little evidence that the same is true for males. In females, testosterone influences thresholds in both a frequency-dependent, and a stimulus-dependent manner.

Testosterone may decrease the frequency at which females are most sensitive within the BP range. Testosterone-treated females had a trend toward a lower HBF than control females. We found no such effect in testosterone-treated males. This result suggests that circulating steroids may be a factor in HBF tuning in females and may explain some of the discordance between size dimorphism and BP tuning sex differences found in some species. The role of testosterone in HBF tuning requires further investigation to establish the magnitude of the effect. In this study we sampled at every

200 Hz within the HBF range and sampling at a higher spectral resolution might produce a clearer picture of the range of variation within each group. Additionally, single unit studies in the VIIIth nerve could reveal whether the influence of testosterone occurs in the auditory periphery.

Despite the lack of evidence for a shift in thresholds at any of the sensitivity peaks in testosterone-treated animals, subtle changes resulted in shifts in the relative sensitivities between the AP peaks and the BP peak. With these small shifts, thresholds at MBF no longer differed from thresholds at HBF in both males and females. The relative amplitude of the two spectral bands of the male advertisement call does influence female behavior (Gerhardt, 1974, 1976) and a steroid induced shift in the relative sensitivity to those bands may therefore have behavioral consequences. The magnitude of the shifts is small and therefore a behavioral study to determine meaningful differences is necessary to understand whether these shifts in MBF/HBF ratios actually result in differences in behavior.

For males, testosterone did not influence audiogram thresholds, regardless of whether the test frequencies fall within the spectral bands of the male advertisement call. The lack of an influence of testosterone manipulation on neural thresholds in males is consistent with results reported previously for multiunit audiograms in *Hyla cinere*a (Penna et al., 1992) where testosterone treatment had no effect in castrated males. In the previous study, gonad removal without steroid replacement did influence audiogram thresholds suggesting that testosterone may require the presence of other gonadal steroids to influence thresholds. Our study verifies that testosterone treatment in the presence of gonads does not influence male thresholds. Given that gonadectomy did influence

thresholds in the previous study, other testicular steroids such as estradiol may play a role, however this has not been tested in males.

In females, testosterone significantly increased audiogram thresholds for frequencies corresponding to the spectral bands of the male advertisement call but not for those outside the advertisement call bands. This demonstrates that for thresholds to pure-tone stimuli, testosterone influences are limited to spectral bands that are likely to be involved in processing communication signals. This result is in contrast to a previous study that demonstrated no influence of testosterone on thresholds in females (Penna et al., 1992). In our study, plasma testosterone levels were analyzed using enzyme immunoassay to confirm that testosterone treatment was effective in elevating testosterone levels whereas the previous study reported no such verification. A difference in the effectiveness of the testosterone treatment may explain the different results between these two studies. Additionally, the testosterone levels achieved by implants in this study are likely higher than the physiological range for this species (Burmeister and Wilczynski, 2000) and thus may be pharmacological. The lack of an influence of testosterone in males but a significant influence in females suggests that the role of testosterone in modulating thresholds differs between the sexes. Further studies must be done to address whether the influence in females is dose-dependent.

The influence of testosterone on auditory thresholds in the TS leads to questions about its site of action within the auditory system. Responses in the TS represent the function of lower auditory nuclei and therefore may be an indication of sex differences and hormone action earlier in the auditory pathway. Considering the presence of steroid receptors within the TS (Dimeglio et al., 1987; Guerriero et al., 2005; Kelley, 1980) and

previous data demonstrating sex differences in VIIIth nerve responses (discussed above) and otoacoustic emissions (Vassilakis et al., 2004) of several anuran species, it is likely that the results of this study are a combination of effects within the TS and earlier processing centers including the ear.

Suggestions for behavioral significance

Auditory processing in the TS is important in perception and phonotactic behavior suggesting that sex differences revealed in this study would influence auditory evoked behavior. Behavioral audiograms, using the modified reflex response, match closely to the multiunit audiograms recorded from the *Hyla cinerea* TS, demonstrating the perceptual relevance of neural thresholds at this level of the brain (Megela-Simmons et al., 1985). Additionally, lesions of the TS in *Hyla versicolor* eliminate phonotactic behavior in two-alternative choice tests while extensive thalamic lesions do not (Endepols et al., 2003).

A sex difference in the relative sensitivities between the AP and BP range of frequencies may influence how we currently understand behavioral responses to the advertisement call in this species. Gerhardt (1974) demonstrated that, behaviorally, females are sensitive to the relative amplitudes of the spectral bands of the male advertisement call. In two-alternative choice tests, a synthetic call with a relative amplitude difference of as little as 10 dB SPL between the low and high frequency bands rendered the call less attractive compared to calls with smaller differences. Complementary tests in males have not been reported. The results of the current study suggest that males may perform differently on a similar type of behavioral task.

Additionally, the relative amplitudes of the two bands of the advertisement call attenuate differentially over distance and this factor influences discrimination behavior in females (Gerhardt, 1976) but the influence in males is not known. Further studies are needed to understand the relationship between the neural representation of the spectral bands and their influence on behavior in both males and females.

Higher neural thresholds to frequencies outside the bands of the male advertisement call in female *H. cinerea* suggest that, in a noisy environment, the female auditory system may filter out frequencies that would interfere with the detection and discrimination of the communication signal. Broadband noise does influence behavioral responses in female *H. cinerea* (Ehret and Gerhardt, 1980). Additionally, behavioral evidence in females of another Hylid species, *H. ebraccata*, suggests that the natural noise of a chorus does indeed impair detection and discrimination (Wollerman, 1999; Wollerman and Wiley, 2002). Experiments comparing males and females on these tasks are needed to test the behavioral implications of audiogram sex differences.

Variation in both female mate choice behavior and testosterone levels across the breeding cycle, in another frog species, is consistent with the effects of testosterone on female thresholds in this study. In female *Physalaemus pustulosus*, testosterone levels are highest just prior to the expression of maximal levels of reproductive behavior and testosterone levels are low when behavioral levels become high (Lynch et al., 2005; Lynch and Wilczynski, 2005). Testosterone likely influences female behavioral thresholds in part through action on forebrain nuclei, however results from this study suggest that testosterone also increases neural thresholds at earlier sensory processing nuclei. Increased thresholds are limited to pure-tone stimuli that are associated with

frequencies found in communications signals, suggesting that testosterone may reduce the filtering capability of the female auditory system. Behavioral examination of testosterone influence on signal detection in a noisy environment is necessary to test this hypothesis.

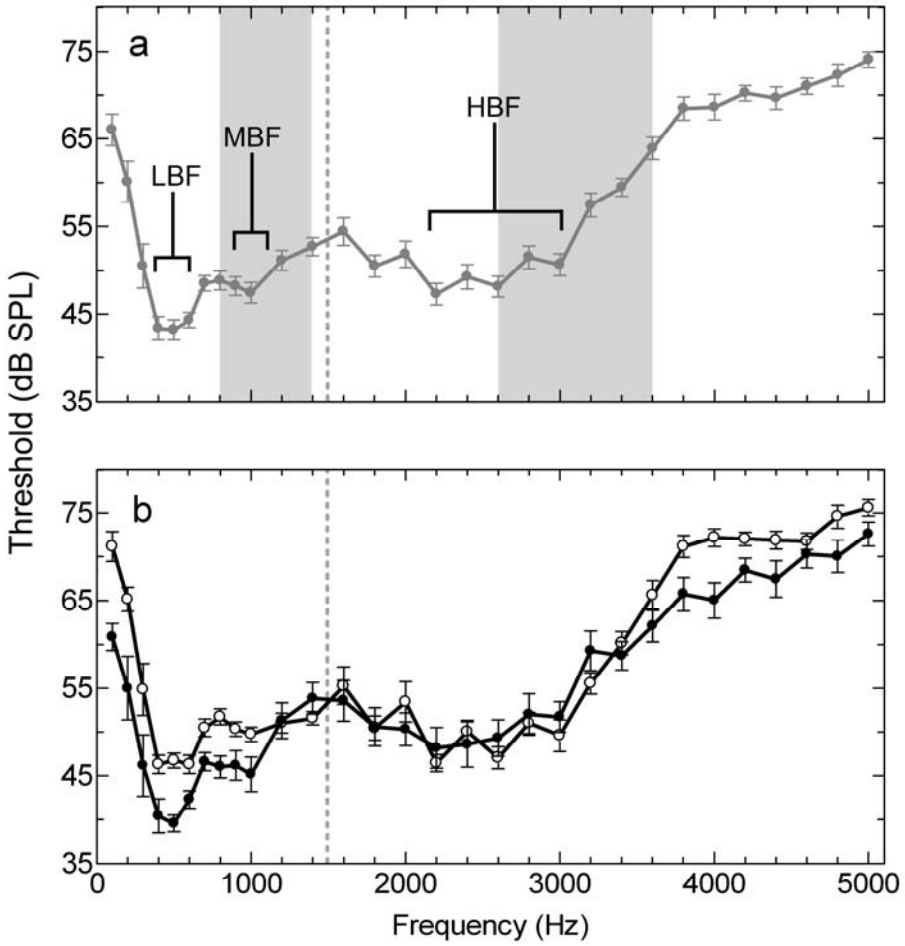

Figure 1. Mean multiunit audiograms from Hyla cinerea. (a) Male and female audiograms combined. Each filled circle represents the mean threshold in response to the corresponding pure tone (n=14). Brackets denote the three peaks in sensitivity and the grey vertical bars denote the spectral bands of the male advertisement call. (b) Males (filled circles, n=7) and females (open circles, n=7) represented in separate audiograms. The vertical dashed line in (a) and (b) represents the division between the AP and BP range of frequencies.

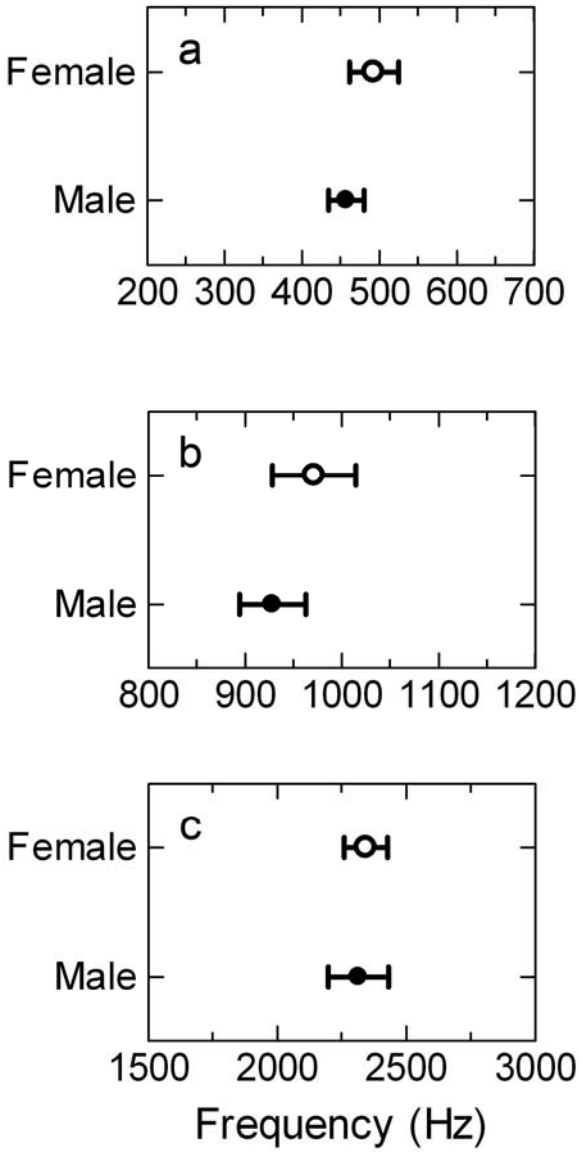

Figure 2. Audiogram best frequencies for females (open circles) and males (filled circles) at (a) LBF, (b) MBF and (c) HBF.

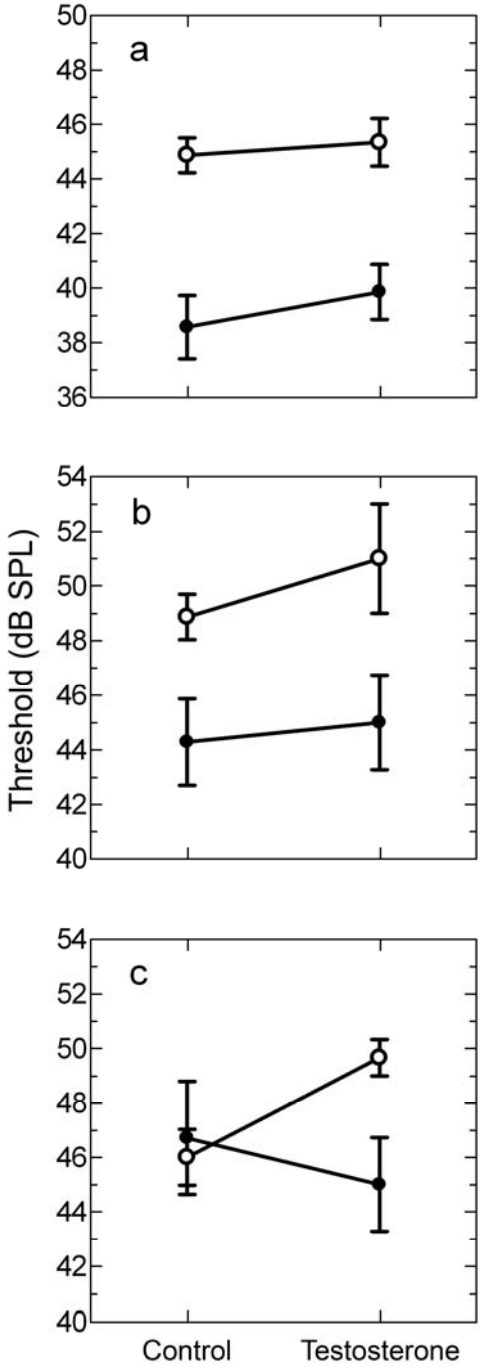

Figure 3. Threshold at audiogram best frequency. Comparison of females (open circles) and males (filled circles) on threshold at (a) LBF, (b) MBF and (c) HBF. Refer to the results section for statistical comparisons of sex and testosterone effects.

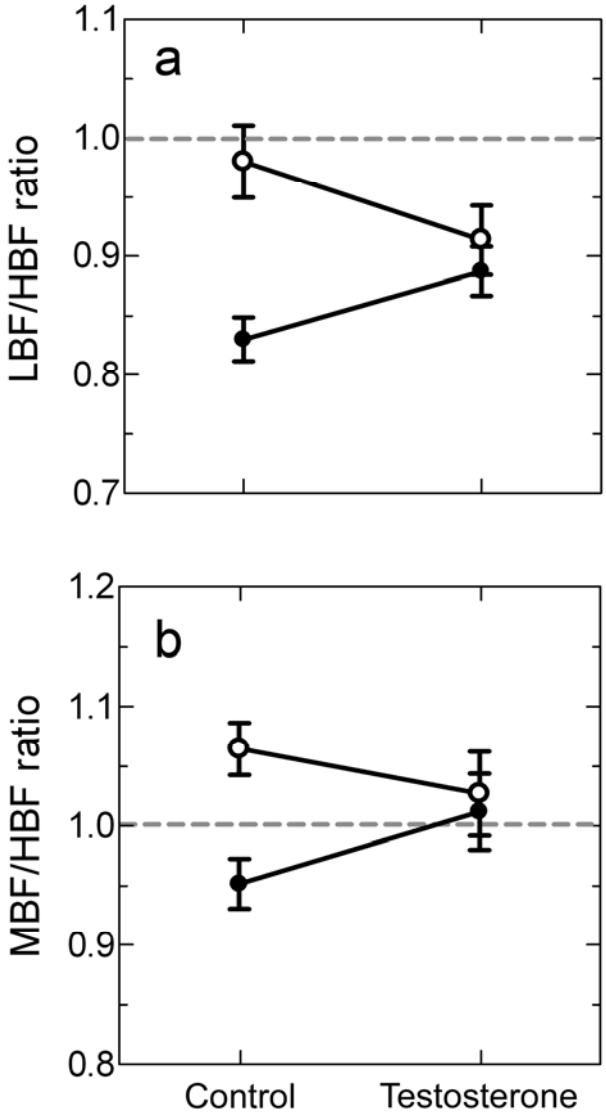

Figure 4. The influence of sex and testosterone on relative thresholds at best frequency for (a) LBF/HBF and (b) MBF/HBF. Females are represented by open circles and males by filled circles. The dashed line denotes the null hypothesis of 1.0 and thus no relative difference in sensitivity between the two peaks being compared. Refer to the results section for statistical comparisons of sex and testosterone effects.

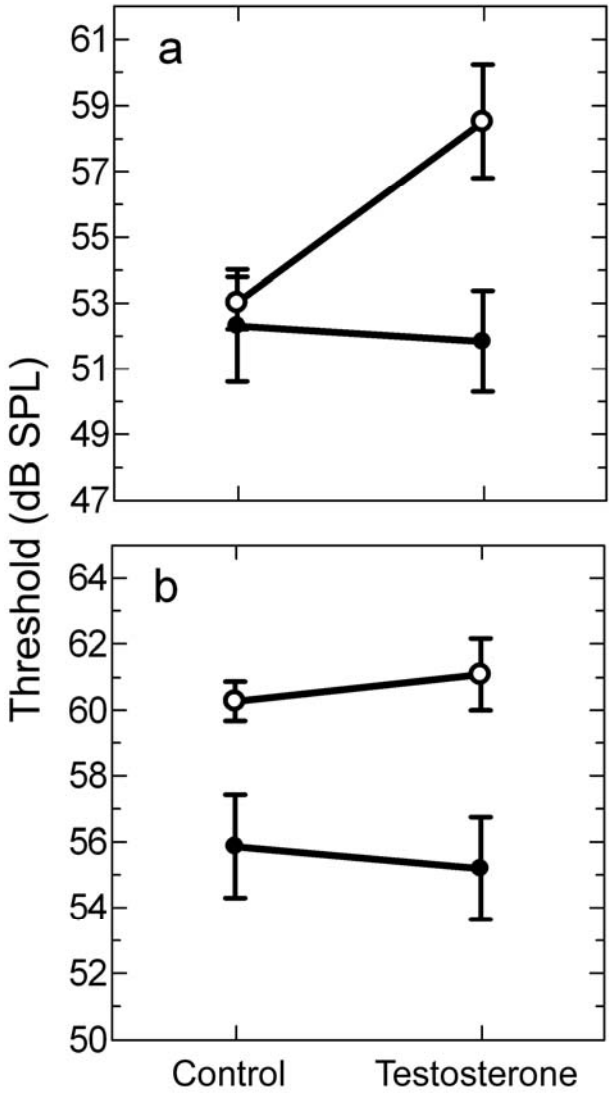

Figure 5. Mean audiogram thresholds based on advertisement call frequencies. Thresholds are collapsed across frequencies on whether they fall (a) within the bands of the advertisement call or (b) outside the range for females (open circles) and males (filled circles). Refer to the results section for statistical comparisons of sex and testosterone effects.

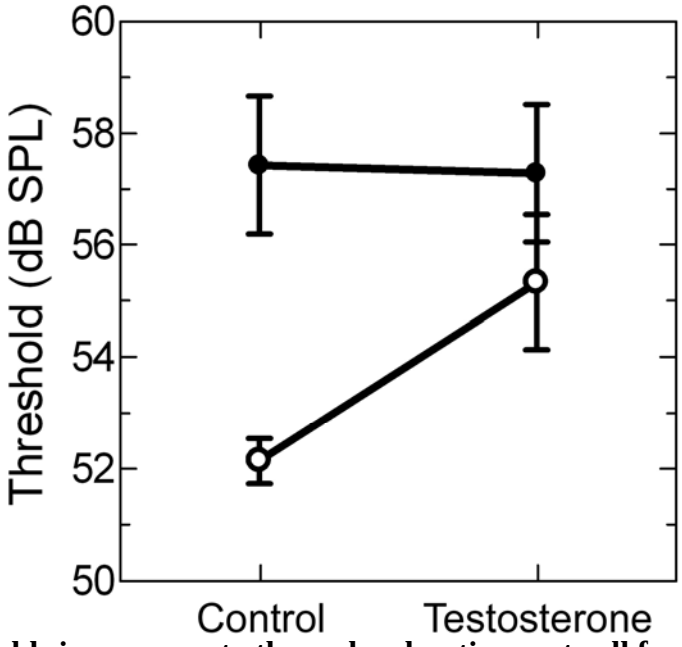

Figure 6. Thresholds in response to the male advertisement call for females (open circles) and males (filled circles). Refer to the results section for statistical comparisons of sex and testosterone effects.

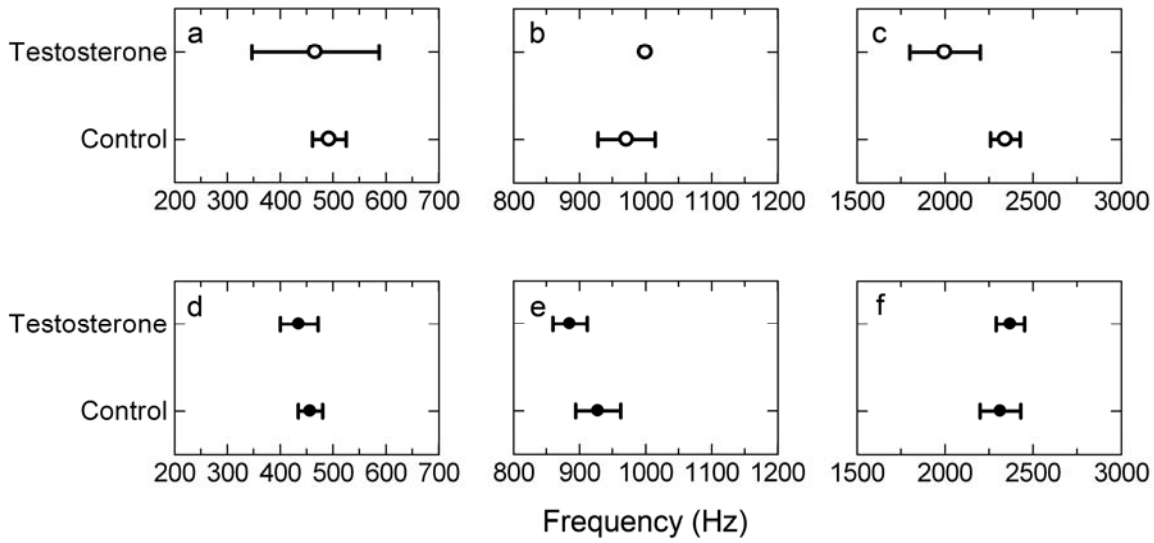

Figure 7. Audiogram best frequency by testosterone treatment for (a, b, c) females and (c, d, e) males. The three best frequencies are (a, c) LBF, (b, d) MBF and (c, e) HBF.

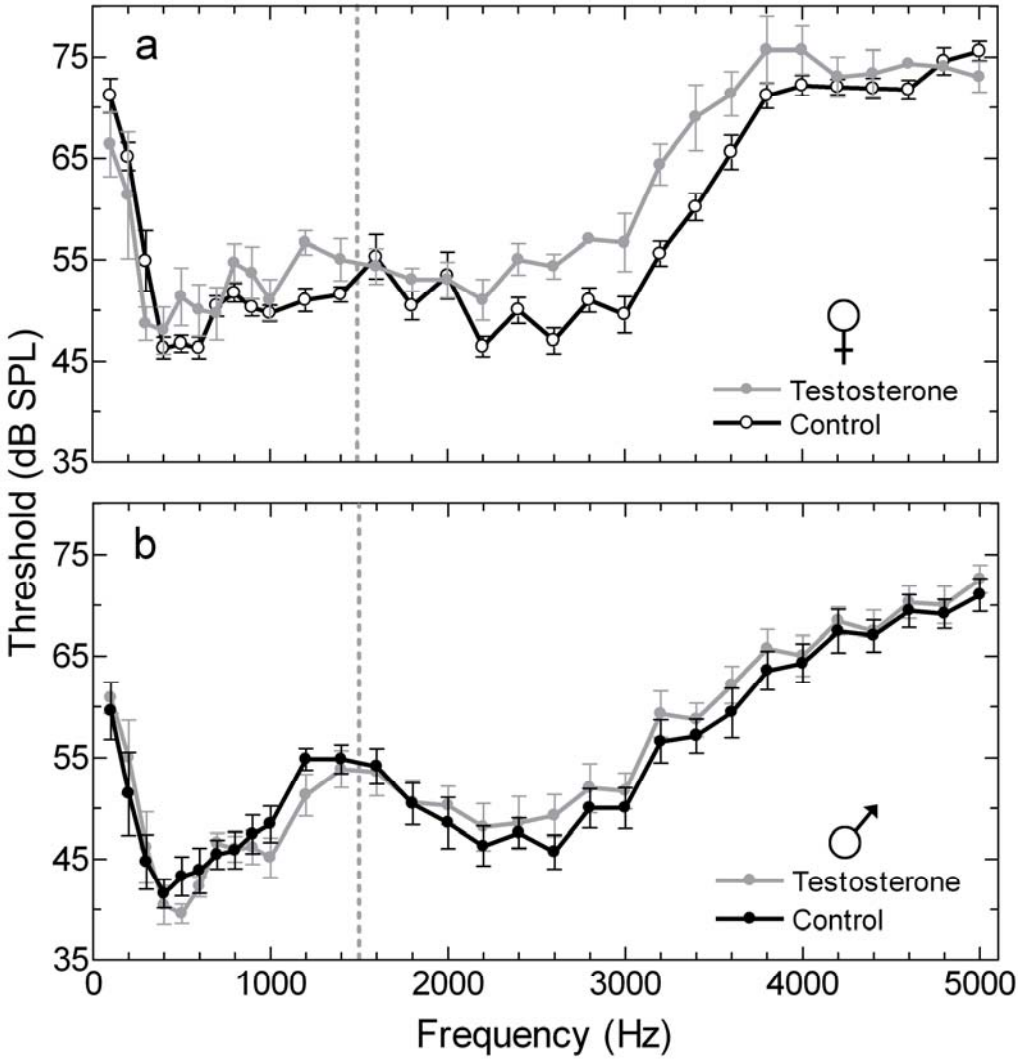

Figure 8. Multiunit audiograms from the TS comparing control and testosterone treatment in (a) females and (b) males. The dashed line divides the AP and BP range of frequencies.

Chapter 3: Facultative sex differences in auditory midbrain response strengths in the green treefrog, *Hyla cinerea*

INTRODUCTION

In many animal communication systems, males produce a signal intended for both female and male conspecifics. These different receivers often produce different behavioral responses to the same stimulus and this phenomenon occurs across animal groups and several sensory modalities (for examples see: Bernal et al., 2007; Martin & Lopez, 2006; Morris & Ryan, 1996; Schmitt et al., 1994; Searcy & Brenowitz, 1988). Additionally, sex differences in the metabolic cost of gamete production often result in males repeatedly mating over short periods of time and females cycling between periods of high and low responsiveness to mating signals (Trivers, 1972). Levels of responsiveness in females typically increase as oviposition/ovulation approaches and reduce after oviposition/ovulation (Bosch & Boyero, 2004; Feinberg et al., 2006; Lacreuse et al., 2007; Lynch et al., 2005; McKibben & Bass, 1998; Moffatt, 2003; Penton-Voak et al., 1999). These individual variations in behavior may be, in part, due to differences in sensory processing of communication signals. The experiments in this study aim to address sex differences and the influence of female reproductive condition on auditory processing at the level of the central auditory system.

The anuran amphibian, a classic model system for vocal communication, exhibits sexual dimorphisms in behavioral responses to the male advertisement call. Sexes differ in the spectral component of the call to which they respond (Narins & Capranica, 1976), in recognition of calls as conspecific (Bernal et al., 2007) and in discrimination among stimuli to which they respond (Schwartz, 1987; Schwartz & Wells, 1985). All of these types of sex differences could result from differences in how the sensory system encodes the auditory stimulus.

Sex differences in frequency selectivity have been demonstrated neurophysiologically based on thresholds in the anuran peripheral auditory system (Keddy-Hector et al., 1992; McClelland et al., 1997; Narins & Capranica, 1976; Vassilakis et al., 2004; Wilczynski et al., 1992; Wilczynski et al., 1984) and the auditory midbrain, the torus semicircularis (TS) (Miranda & Wilczynski, submitted, chapter two this dissertation). Best excitatory frequency and selectivity at threshold often differ significantly when compared to responses to stimuli above threshold (Capranica, 1992; Schwartz & Gerhardt, 1998). Considering that frogs commonly communicate at levels well above the threshold for detecting signals, an understanding of sex differences in auditory processing requires assessment at these suprathreshold stimulus levels.

Anuran females also exhibit variation in reproductive behavior based on the individual's reproductive condition. Previous studies report differences in neurophysiological response properties in the TS between breeding and non-breeding seasons (Goense & Feng, 2005; Hillery, 1984; Walkowiak, 1980). Female anurans also show clear evidence of condition-dependent mating behavior within a single breeding season although, to our knowledge, no study has ever addressed plasticity in the auditory

system on this shorter time scale. Female *Hyla cinerea* fail to respond to male mating calls after oviposition (Gerhardt, 1974). Preventing oviposition by refrigeration until behavioral testing, maintains normal mating responses until oviposition occurs. Reproductive condition also influences mate choice decisions in other anuran species (Bosch & Boyero, 2004; Lea et al., 2000; Lynch et al., 2005). While such condition-dependent behavior is likely due, in part, to neural mechanisms in auditory forebrain regions, questions still remain about whether plasticity in earlier auditory processing mechanisms also contribute.

The TS is a site at which the effects of sex and reproductive state may significantly influence the processing of communication signals. First, the TS integrates the vast majority of ascending auditory inputs between the brainstem auditory nuclei and the forebrain (Wilczynski & Endepols, 2007). This integration results in a specialization within the TS for processing complex stimuli, such as communication signals, in addition to the more simple pure-tone stimuli (Feng and Ratnam, 2000; Rose and Gooler, 2006). Second, auditory processing in the TS is important in perception and phonotactic behavior. Behavioral audiograms, using the modified reflex response, match closely to the multiunit audiograms recorded from the *Hyla cinerea* TS (Megela-Simmons et al., 1985). Lesions of the TS in *Hyla versicolor* also eliminate phonotactic behavior in two-alternative choice tests while extensive thalamic lesions do not (Endepols et al., 2003). Last, the TS is sensitive to steroid hormones with evidence for receptors reported in *Xenopus laevis* (Kelley, 1980) and *Rana esculenta* (Dimeglio et al., 1987; Guerriero et al., 2005).

Evidence suggests that sex and reproductive state differences may be limited to particular spectral bands. The inner ear of the anuran contains two physically and functionally different sensory end-organs that process different spectral bands of airborne auditory stimuli (Smotherman & Narins, 2000). The amphibian papilla (AP) is a tonotopic structure that responds to a spectral band of approximately 100-1400 Hz for *H. cinerea*. The basilar papilla (BP) is an end-organ that responds at a single best excitatory frequency and processes frequencies in the range between 1600-5000 Hz. The male advertisement call of *H. cinerea* contains two spectral peaks, one falling within the AP spectral band and the other within the BP band. The relative amplitude of the two peaks is important in eliciting female phonotaxis (Gerhardt, 1974). Neural responses in male *H. cinerea* are more sensitive than females at the sensitivity peaks of the AP but are not different from females at the BP peak (Miranda & Wilczynski, submitted, chapter two this dissertation). The responses of auditory systems are not linear across stimulus amplitudes (Moore, 2003) and close-range communication in *H. cinerea* occurs well above neural threshold (Gerhardt, 1974). For these reasons it is unknown whether frequency-specific sex differences exist at levels consistent with close-range communication.

Sex and reproductive state also may influence the auditory system in a stimulus-specific manner. A neuron's responses to pure tones, noise bursts and conspecific advertisement calls are not related linearly (Eggermont et al., 1983; Rose & Capranica, 1983; Rose & Capranica, 1985; Theunissen et al., 2000; Woolley et al., 2006), and individual differences in the neural response may not be universal across stimuli. Additionally, the matched filter hypothesis suggests that dominant frequencies in the

male advertisement call match the peak sensitivities of the auditory system (Capranica, 1978). If sex and reproductive state only influence multiunit responses at the sensitivity peaks of the auditory system, we may expect to see differences only in response to the advertisement call and not to a broadband noise stimulus.

In this study we tested whether males and females differ in multiunit response strengths to auditory stimuli and whether sex differences depended on the reproductive state of the female. We also tested whether sex and reproductive state differences were frequency-specific and stimulus-specific.

MATERIALS AND METHODS

Animal collection and care

Male and female *Hyla cinerea* were captured at Brackenridge Field Laboratory (Austin, TX) between June and August of 2006. Animals were captured by hand at small ponds, in PVC refugia placed near the edge of the ponds (Boughton et al., 2000), and on a shed near one of the ponds. Four of 10 females were in amplexus with a male conspecific upon capture. After capture, animals were taken to the lab and housed individually in 12”W x 8”D x 7.5”H aquaria for 3-6 days. Three out of the four females found in amplexus spontaneously released eggs within 24 hours of capture. Based on these behavioral characteristics we assigned animals to the following groups: a) male (n=10), b) unamplexed female (n=6) and c) postmated female (n=4). We fed the frogs crickets *ad libitum* and provided water in a bowl inside each aquarium. Environmental conditions were 23ºC and 14:10 light:dark cycle. All procedures were performed in

accordance with a protocol approved by The University of Texas at Austin Institutional Animal Care and Use Committee.

Neurophysiology preparation

Methods for preparing the animal for electrophysiology recordings were described previously by Wilczynski et al. (1993). Within 72 hours of capture we anesthetized animals in a solution of 0.1% tricane methyl sulfonate (Sigma, St. Louis, MO) brought to a pH of 7.3 with $NaHCO_3$. A section of skin was cut and folded back to expose the brain case covering the midbrain. A small piece of brain case covering the optic tectum was cut away and replaced with a damp piece of tissue. The skin was replaced over the exposed area and a small amount of tissue glue was used to seal the skin. Animals were allowed to recover for 2-5 days in their own holding aquarium. On the day of the electrophysiological recordings, animals were immobilized with an intramuscular injection of curare (d-tubocurarine chloride; 10mg/g body weight) and 2% lidocane was applied as a local anesthetic to the tissue surrounding the exposed site. Recording took place in an Industrial Acoustics sound-attenuating chamber with the animal draped in wet paper towels at an ambient temperature of 23°C.

Acoustic stimuli

We presented sound to the ear of the animal through an insert earphone (Etymotic Research model ER-2, Elk Grove Village, IL) with a custom adapter to create a closed field system. We used a Brüel & Kjær Precision Integrating Sound Level Meter Type 2230 to measure the response of the earphone system between 100-5000 Hz and

determine the stimulus amplitude at the animal's ear. Earphone calibration was controlled by SigGen32 software (Tucker Davis Technologies (TDT), Alachua, FL, v.3.5,) to ensure a flat frequency response at the frog's ear. In order to determine a spike rate function, all stimuli were presented at 60, 70, 80, 90 and 100 dB SPL.

Acoustic stimuli consisted of noise bursts and male advertisement calls (detailed below). Noise and advertisement call stimuli were presented in the form of three different categories based on spectral bandwidth: 1) a full frequency category was bandpass filtered from 100-5000 Hz to correspond with the known hearing range of *H. cinerea*, 2) an AP frequency category was bandpass filtered from 100-1400 Hz to correspond with the sensitivity of the amphibian papilla and 3) a BP category was filtered from 1600-5000 Hz to correspond with the sensitivity of the basilar papilla.

Noise bursts were designed in SigGen32 and were 180 ms in duration with a 5 ms $\cos^2$ onset gating function. To reduce the number of stimuli yet maintain variation in the relative amplitudes of individual frequencies contained within the noise across presentations, a new noise sample was generated by the software for each repeated presentation. All noise stimuli were presented in one block and presented in random order until each bandwidth-amplitude combination was presented 20 times.

We obtained focal recordings of natural calls from individuals at ponds in Harris County, GA (1 individual), Fulton County, GA (2 individuals) and Travis County, TX (4 individuals) (advertisement call example, Figure 9). We recorded calls to audio cassette with a Marantz PMD 420 using a Marantz EC-7 or a Sony ECM-MS907 microphone. The calls were digitized at 11025 Hz and bandpass filtered to correspond with the three stimulus categories using Raven sound analysis software (Charif et al., 2004). All

stimulus presentation was controlled by Brainware (TDT, v.7.301). Natural stimuli were presented in three blocks of 35-40 different stimuli in order to allow for periodic assessment of recording stability between blocks. Among different recording sites in each animal, blocks were presented in random order. Within each block, stimuli were presented randomly until 20 repetitions of each stimulus were presented.

Neurophysiology

Acoustically stimulated extracellular multiunit responses were obtained from the TS contralateral to the earphone using glass micropipettes. We pulled sharp electrodes with a long taper on a horizontal puller and then broke the tip to a diameter of 20-30 μm. We filled the electrodes with 1M NaCl. The extracellular response was first amplified by a Grass Instruments P15 differential AC preamplifier (West Warwick, RI), then an A-M Systems Model 3000 differential AC/DC amplifier (Sequim, WA) and bandpass filtered between 300-5000 Hz. The response was then digitized at 25 kHz (TDT, model AD1) and stored on a personal computer. Spike counting was done by Brainware set to the artifact rejection trigger mode. We set the trigger level for spike counting at ± 4 standard deviations with respect to background activity levels as assessed in an area adjacent to the recording site where no spontaneous spikes were detected. We identified the TS by its robust response to a search stimulus consisting of two tones (900 Hz and 3000 Hz) presented simultaneously at a peak amplitude of 80 dB SPL. We collected data from up to three sites per animal in order to account for any tonotopic pattern in response properties. The medial-lateral location of each recording site was noted. Medial lateral location did not show any pattern of influence on response strengths within or among

animals. Therefore, data from all sites within an animal were combined to represent the TS response to auditory stimuli.

Data analysis

The magnitude of a response to a given stimulus was calculated as response strength normalized to baseline spontaneous activity. Spontaneous activity was quantified as the number of spikes that occurred during a period of silence preceding the presentation of the stimulus. The time window for the spontaneous spike count matched the window used for counting spike activity during the stimulus. Response strength was defined as a *z*-score as described previously (Coleman & Mooney, 2004). The *z*-score subtracts the mean number of spikes during the baseline period from the mean number of spikes during the stimulus presentation and divides that by the standard deviation of that difference over 20 stimulus presentations. The calculation is as follows:

$$z = \frac{\overline{S} - \overline{B}}{\sqrt{\mathrm{Var}(S) - \mathrm{Var}(B) - 2\mathrm{Covar}(S,B)}},$$

Where $\overline{S}$ is the mean response during the stimulus presentation, $\overline{B}$ is the mean response during a baseline period preceding the stimulus and the denominator is the standard deviation of $(S - B)$.

To test whether the experimental groups differed in baseline spontaneous activity, we quantified the spike rate during a period of silence preceding the presentation of two of the auditory stimuli: a) the full frequency version of the noise burst presented at 70 dB SPL and b) the full frequency version of one of the advertisement calls (Harris County

call) presented at 70 dB SPL. These two stimuli were chosen because they were part of two separate stimulus blocks and therefore represent two time points during recording from a site in the TS. This resulted in a sample size of 40-120 counts per animal. The mean spontaneous spike count for each animal was then used as a score to represent spontaneous activity. These scores were then used for statistical comparison of the experimental groups.

Statistical analysis

We conducted all statistical analyses using SPSS 14.0 for Windows. We used principal component analysis to create a linear combination of a single animal's z-scores in response to all seven of the representative advertisement calls. For example, a single component was extracted to describe an animal's response strength to all seven of the full advertisement calls at 60 dB SPL, another component at 70 dB SPL and so on. The same was done for responses to the AP bandpass filtered advertisement calls and BP bandpass filtered advertisement calls. The first component was a very good descriptor of an animal's combined response to the corresponding stimulus category (for a representative scree plot, see Figure 10). The first component captured a maximum of 96.04% and a minimum of 72.25% of the variance in responses to a given stimulus, with a median of 84.05%. Unstandardized component scores were then computed for statistical analysis and graphical representation.

Repeated measures ANOVA with a between-subject factor of reproductive status (male, unamplexed female and postmated female) was used to compare groups on their response strengths. Separate analyses were conducted for each of the following

categories of stimuli: full noise, AP band-limited noise, BP band-limited noise, full advertisement call, AP band-limited advertisement call and BP band-limited advertisement call. For responses to noise stimuli, the dependent variable was the response strength (z-score) which was tested at the five stimulus amplitudes. For responses to the advertisement call stimuli, the dependent variable was the unstandardized principle component score of the response strengths which was also tested at the five stimulus amplitudes. The data violated the assumption of sphericity for repeated measures ANOVA, and therefore the degrees of freedom for these analyses were adjusted using the Huynh-Feldt correction. When a significant main effect of reproductive status or an interaction between reproductive status and stimulus amplitude was detected, we conducted Bonferroni corrected post-hoc analyses to test the between-subjects comparisons. We also used ANOVA to compare males, unamplexed females and postmated females at each stimulus amplitude.

Lastly, we used ANOVA with Welch's F statistic to compare groups on spontaneous activity. A p-value for statistical significance was set at $p < 0.05$ for all tests in this study.

RESULTS

Spontaneous activity

Data for multiunit spontaneous activity failed to meet the assumption of homogeneity of variances among groups [Levene statistic, $F(2,17)=5.048$, $p=0.019$]. Spontaneous activity was not significantly different among males, unamplexed females

and postmated females [Welch's F(2, 8.51)= 2.841, p=0.113]. Mean spontaneous spike rate for males was 36.9 ± 7.9 spikes/second (mean ± SE), 16.9 ± 3.0 spikes/second for unamplexed females and 23.4 ± 5.2 spikes/second for postmated females (Figure 11a) Additionally, spontaneous activity scores did not correlate with z-scores [example: z-scores for response to AP bandpassed noise at 60 dB SPL, r (20) = 0.052, p = 0.828] (Figure 11b).

Response to band-limited noise

Reproductive status significantly influenced response strengths to the AP band-limited noise stimulus in a stimulus amplitude-specific manner with a significant interaction between stimulus amplitude and status [F(5.46, 46.40)= 3.050, p=0.016] (Figure 12b). Postmated females had significantly lower response strengths overall, when compared to unamplexed females (t(17)= 2.15, p=0.046) as well as males (t(17)= 2.77, p=0.013). Reproductive status significantly influenced response strengths at 60 dB SPL [F(2, 17)= 4.172, p=0.034], 70 dB SPL [F(2, 17)= 7.058, p=0.006] 80 dB SPL [F(2, 17)= 6.689, p=0.007] and 90 dB SPL [F(2, 17)= 3.999, p=0.038] but not 100 dB SPL [F(2, 17)= 0.232, p=0.796] (Figure 12b).

Reproductive status did not significantly influence response strengths to the BP band-limited noise stimuli [F(2, 17)= 0.229, p=0.798] or the full frequency noise stimuli [F(2, 17)= 2.238, p=0.137] (Figure 12a, c).

Response to the advertisement call

Reproductive status showed a trend toward an influence on response strengths to the AP band-limited advertisement call stimulus [$F(2, 17)= 2.869$, $p=0.084$] (Figure 13) with no significant interaction between status and stimulus amplitude. Postmated females had a trend toward lower response strengths overall, when compared to males ($t(17)= 1.72$, $p=0.104$) but not unamplexed females ($t(17)= 1.40$, $p=0.179$). Reproductive status significantly influenced response strengths at 60 dB SPL [$F(2, 17)= 3.858$, $p=0.042$] and 70 dB SPL [$F(2, 17)= 3.866$, $p=0.041$] and showed a trend toward an influence at 80 dB SPL [$F(2, 17)= 2.771$, $p=0.091$]. Reproductive status did not significantly influence response strengths at 90 dB SPL [$F(2, 17)= 2.030$, $p=0.162$] or 100 dB SPL [$F(2, 17)= 1.567$, $p=0.237$] (Figure 13b).

Reproductive status did not significantly influence response strengths to the BP band-limited advertisement call stimuli [$F(2, 17)= 0.320$, $p=0.731$] or the full frequency noise stimuli [$F(2, 17)= 0.288$ $p=0.754$] (Figure 13a, c).

DISCUSSION

Our goal for this study was to determine whether male and female *H. cinerea* differed in neurophysiological responses to auditory stimuli at suprathreshold levels consistent with close-range communication. We tested whether sex differences in the TS were frequency-dependent based on spectral bands associated with sensitivities in the peripheral auditory system. We also tested whether differences were stimulus-dependent

based on the behavioral relevance of the stimulus. Our results demonstrate sex differences that are apparent only when considering the reproductive status of females. Postmated females had significantly lower response strengths to both noise and advertisement call stimuli when compared to unamplexed females as well as males. Males and unamplexed females were not significantly different. Reduced response strengths were limited to the lower frequency range corresponding with the AP of the peripheral auditory system.

Sex differences in response to the AP range of frequencies in this study, are consistent with a previous study on multiunit thresholds in *H. cinerea* (Miranda, chapter two this dissertation). In the previous study, male thresholds to pure tones were significantly lower than female thresholds at both of the two sensitivity peaks in the AP range of frequencies but not at the BP peak. Lower thresholds (higher sensitivity) would predict greater responses at successively higher amplitudes, until a point of saturation is reached. Female reproductive state in the previous study was not assessed, although the results presented here suggest that females in the previous study may have been in a post-reproductive state. This point cannot be assumed because neural and behavioral responses across a range of stimulus intensities are not linear (Moore, 2003). For this reason, a study of the plasticity of neural thresholds in the AP spectral band with respect to female reproductive condition is needed. The sex difference in the current study held true for both the advertisement call and noise stimuli, which is also consistent with thresholds in the previous study. Male thresholds in the AP range were not limited to the audiogram sensitivity peaks in the previous study, but were lower at almost all of the tested frequencies within the AP range.

Although postmated females had reduced responses to low frequency stimuli, they did not differ significantly from males or unamplexed females in spontaneous activity. Given the small sample sizes, however, we cannot conclude that all groups are the same, only that we cannot support the hypothesis that there are differences. Previous reports of plasticity in spontaneous activity are mixed. Walkowiak (1980) reported encountering a greater number of spontaneously active cells during the breeding period in *Bombina bombina* although no sex comparison was reported and females were recorded from during only the non-breeding period. Goense and Feng (2005) report no seasonal difference in spontaneous activity in male *Rana pipiens pipiens* and females were not considered in that study. The lack of a correlation between spontaneous activity scores and response strengths in our study supports the interpretation that the differences in response strengths that we see are a stimulus-evoked phenomenon and not a result of differences in baseline neural activity.

Steroid hormones are likely candidates to modulate plasticity in female auditory processing across reproductive conditions. These hormones clearly relate to reproductive condition in anurans (Harvey et al., 1997; Licht et al., 1983; Lynch & Wilczynski, 2005; Pierantoni et al., 1984). Activation of the hypothalamic-pituitiary-gonadal axis influences female mate choice and activity-dependent gene expression in the TS of *Physalaemus pustulosus* (Lynch et al., 2005; Lynch & Wilczynski, in press). Additionally, steroid treatment has been demonstrated to influence auditory responses in other anuran species. Estradiol treatment increased auditory evoked potentials in the TS of female *Rana pipiens* in response to pure tones representing the dominant frequencies in the conspecific male advertisement call and at levels consistent with communication

(Yovanof & Feng, 1983). It remains unknown whether estradiol affects auditory processing across all frequencies or just those associated with communication. In another study, testosterone increased thresholds to pure tones for frequency bands corresponding to the male advertisement call but not to frequencies outside the advertisement call bands in female *H. cinerea* (this dissertation, chapter 2). The role of testosterone on auditory responses, above threshold, is unknown and needs further investigation.

The reduction of neural responsiveness to stimuli within the AP spectral band in postmated females may have behavioral effects associated with female mating behavior. The *H. cinerea* male advertisement call has two spectral peaks, one falling within the AP spectral band and the other falling within the BP band. Females are behaviorally sensitive to the relative amplitudes of the spectral peaks of the advertisement call (Gerhardt, 1974). In two-alternative choice tests, a synthetic call with a relative amplitude difference of as little as 10 dB SPL between the low and high spectral peaks rendered the call less attractive compared to calls with smaller differences. Gravid females fail to respond to the male advertisement call after oviposition. A significant reduction in the neural responsiveness to one of the spectral bands may result in a perceptual shift that renders a previously attractive call unattractive to a female that has already mated. In support of this hypothesis, the relative amplitudes of the two peaks of the advertisement call attenuate differentially over distance, and this factor influences discrimination behavior in females (Gerhardt, 1976). At low stimulus levels, females discriminate against a call with an attenuated low spectral peak but do not discriminate against a call with an attenuated high spectral peak. The opposite is true at high stimulus levels. This phenomenon is also in line with our result in which the largest reductions in

response strength occur at the lowest stimulus amplitudes but not at the highest. A detailed behavioral analysis with females in different reproductive conditions is needed to determine whether a shift in the neural representation of this social signal would result in changes in recognition or discrimination.

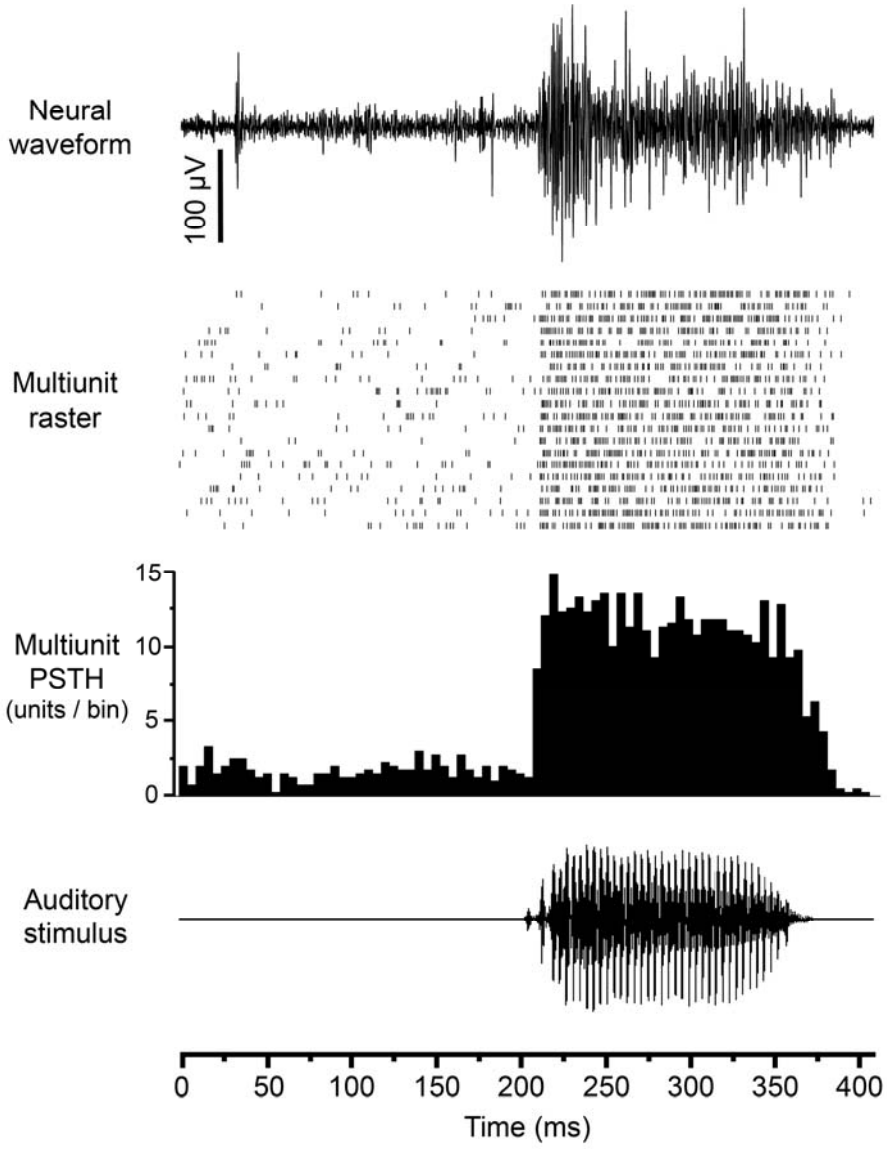

Figure 9. Auditory response in the torus semicircularis of Hyla cinerea. The top trace represents the neural waveform during and preceding the presentation of a male advertisement call. Below the neural waveform is the multiunit raster for 20 presentations of the same advertisement call stimulus and the post-stimulus time histogram (PSTH) for all 20 presentations (bin size 5 ms). Below the histogram is the advertisement call waveform.

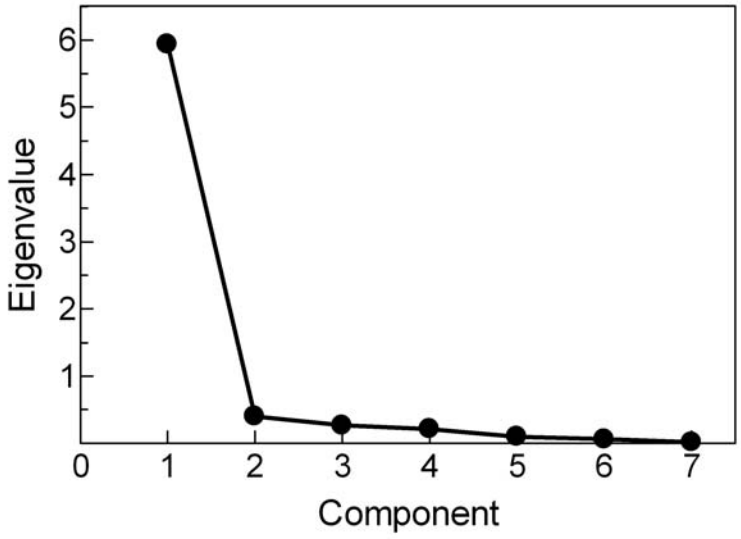

Figure 10. A representative scree plot from a principal component analysis of response strengths (n=20 animals) to the seven advertisement call stimuli presented at 80 dB SPL. Component number one captured a majority of the variance for all stimulus categories. For this example component number one captured 84.87% of the variance.

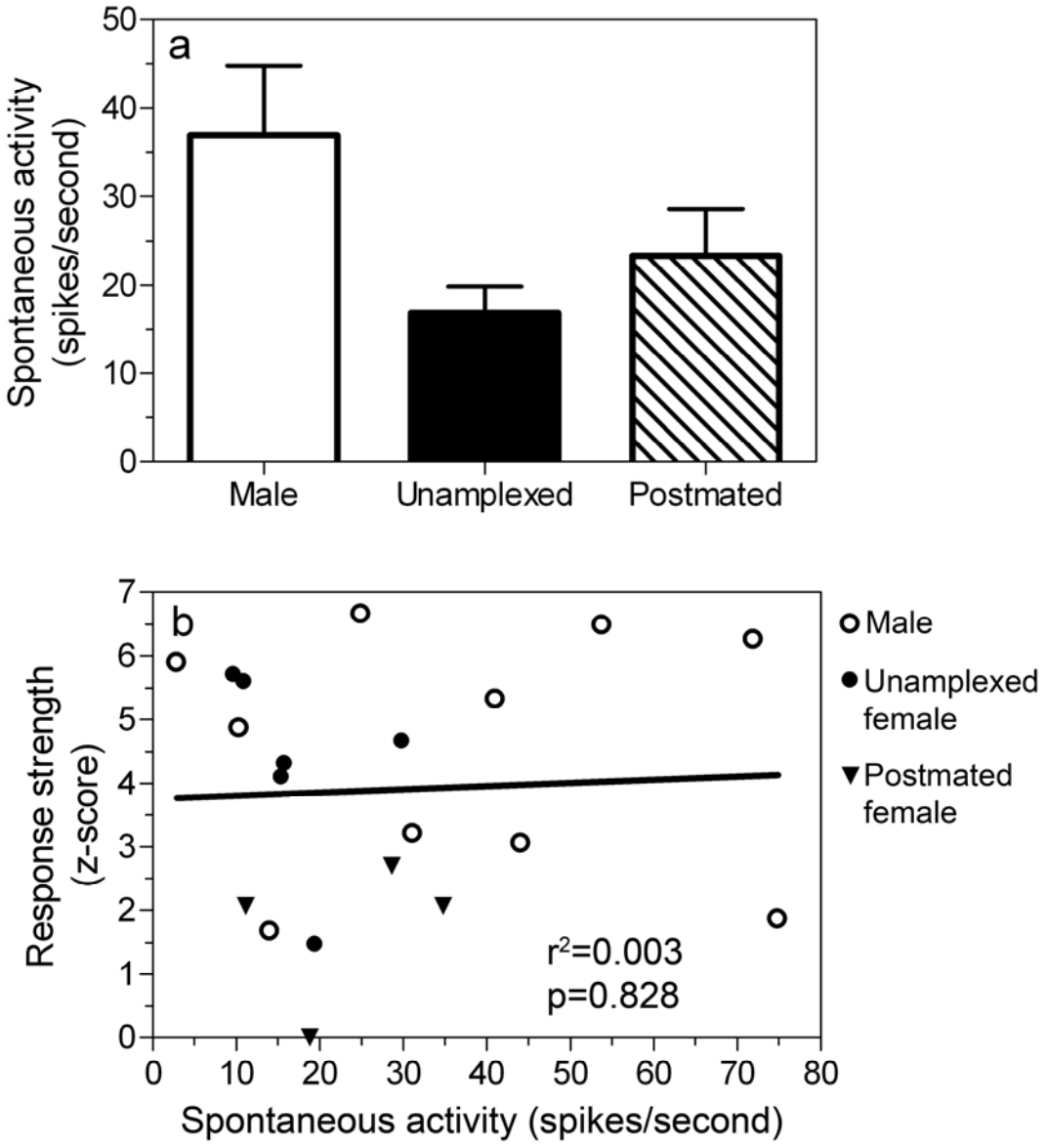

Figure 11. Spontaneous spike rate in the absence of auditory stimulation. (a) Spontaneous activity scores were not significantly different across groups based on sex and female reproductive status. (b) Spontaneous activity scores did not significantly correlate with z-scores. This example plots spontaneous activity scores against z-scores in response to the AP bandpassed noise stimulus presented at 60 dB SPL.

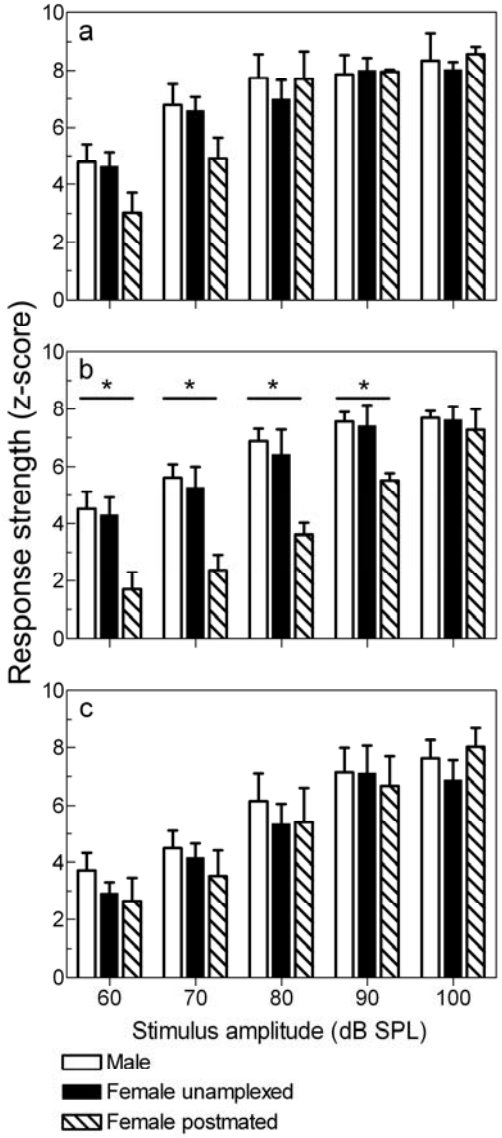

Figure 12. Torus semicircularis response strengths to noise. The multiunit response is presented as a normalized response strength (z-score, calculated for each individual animal in response to each stimulus type). Each bar represents the mean z-score for the corresponding group at the stimulus amplitude for: (a) noise bandpass filtered to encompass the airborne hearing range of 100-5000 Hz, (b) noise filtered to correspond to the AP range of 100-1400 Hz and (c) the BP range of 1600-5000 Hz. Repeated measures ANOVA across stimulus amplitudes is significant in the AP range only ($p<.05$) and in response to all but the highest stimulus amplitudes. Bars with asterisks denote stimulus amplitudes with a significant ANOVA for reproductive status ($p<.05$).

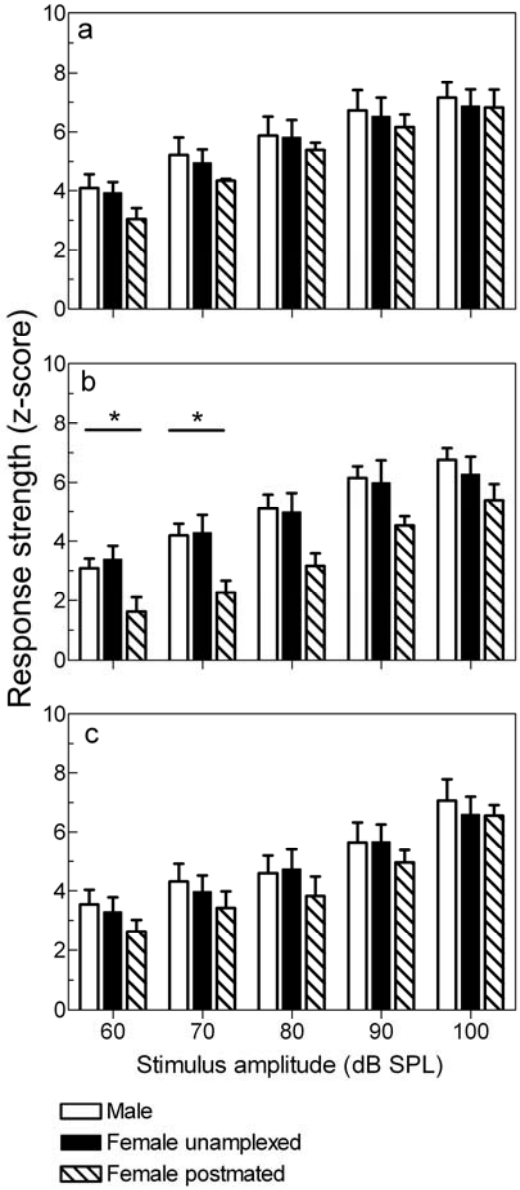

Figure 13. Torus semicircularis response strengths to conspecific advertisement call. The multiunit response is presented as in the noise figure except that each bar represents the mean unstandardized score from a factor analysis on the response to advertisement calls from seven different male *Hyla cinerea*. (a) advertisement call bandpass filtered to encompass the airborne hearing range of 100-5000 Hz, (b) call filtered to correspond to the AP range of 100-1400 Hz and (c) the BP range of 1600-5000 Hz. A trend toward significance in repeated measures ANOVA across stimulus amplitudes occurs in the AP range only (p=.084) and is significant in response to only the lower stimulus amplitudes. Bars with asterisks denote stimulus amplitudes with a significant ANOVA for reproductive status (p<.05).

Chapter 4: Androgen and gonadotropin influences on auditory processing of communication signals in the green treefrog, *Hyla cinerea*

INTRODUCTION

In animal communication, males commonly produce advertisement signals to attract females for reproduction. Females show a great deal of individual variation in behavioral responses to advertisement signals, both among individuals within a species and within an individual over time (Jennions & Petrie, 1997). This variation in behavior is often condition dependent based on reproductive state (Bosch & Boyero, 2004; Feinberg et al., 2006; Lacreuse et al., 2007; Lynch et al., 2005; McKibben & Bass, 1998; Moffatt, 2003; Penton-Voak et al., 1999). Evidence is building to suggest that these individual differences in behavior are in part due to variation in the sensory processing of communication signals (Keller et al., 1986; Maney et al., 2006; Miranda, chapter three this dissertation; Moffatt, 2003; Sisneros & Bass, 2003). In this study, we investigate a proximate mechanism of variation in the auditory processing of vocal signals.

Female anurans use conspecific male advertisement calls in mate choice and show condition-dependent mating behavior based on reproductive state (Bosch & Boyero, 2004; Lea et al., 2000; Lynch et al., 2005). Much of the individual variation in behavior is linked to steroid hormone levels (Gobbetti & Zerani, 1999; Harvey et al., 1997; Lynch & Wilczynski, 2005; Medina et al., 2004). For example, female anurans exhibiting a

heightened level of reproductive behavior and an increased probability of oviposition have elevated levels of estradiol. In contrast, prior to ovulation females show reduced levels of reproductive behavior and elevated levels of androgens. If steroid hormones modulate behavior through action on the auditory system, we predict that elevated estradiol would correspond with enhanced processing of communication stimuli and elevated androgens would correspond with reduced auditory responses.

The auditory midbrain, the torus semicircularis (TS), is a likely area for reproductive hormones to influence the processing of vocal signals. First, the TS integrates the vast majority of ascending auditory inputs between the brainstem auditory nuclei and the forebrain (Wilczynski & Endepols, 2007). This integration results in a specialization within the TS for processing complex stimuli, such as communication signals, in addition to the more simple pure-tone stimuli (Feng & Ratnam, 2000; Rose & Gooler, 2006). Second, auditory processing in the TS is important in auditory detection and phonotactic behavior. Behavioral audiograms match closely to neural audiograms recorded from the TS in several anuran species including *Hyla cinerea* (Bibikov & Elepfandt, 2005; Elepfandt et al., 2000; Megela-Simmons et al., 1985). Lesions of the TS in *Hyla versicolor* also eliminate phonotactic behavior in two-alternative choice tests while extensive thalamic lesions do not (Endepols et al., 2003). Last, the TS is sensitive to steroid hormones with evidence for receptors reported in *Xenopus laevis* (Kelley, 1980) and *Rana esculenta* (Dimeglio et al., 1987; Guerriero et al., 2005).

The organization of the auditory system provides for hypotheses about how hormones may influence auditory processing. First, hormone influences may be limited to particular spectral bands within the hearing range of the animal. The inner ear of the

anuran contains two physically and functionally different sensory end-organs that process different spectral bands of airborne auditory stimuli (Smotherman & Narins, 2000). The amphibian papilla (AP) is a tonotopic structure that responds to a spectral band of approximately 100-1400 Hz for *H. cinerea*. The basilar papilla (BP) is an end-organ that responds at a single best excitatory frequency, the high best frequency (HBF), and processes frequencies in the higher range, between 1600-5000 Hz. Male *H. cinerea* are more sensitive than females at the sensitivity peaks of the AP but are not different from females at the BP peak (Miranda & Wilczynski, submitted, chapter two this dissertation). Second, the matched filter hypothesis suggests that dominant frequencies in the male advertisement call match the peak sensitivities of the auditory system (Capranica, 1978). If steroid hormones only influence multiunit responses at the sensitivity peaks of the auditory system, we may expect to see differences only in response to the advertisement call and not to a broadband noise stimulus.

Some experimental evidence points to a role for steroid hormones in modulating auditory processing of communication signals in the TS. Intracranial ventricular injection of estradiol in female *Rana pipiens* increases the amplitude of auditory evoked potentials in response to all of four different pure-tone stimuli (Yovanof & Feng, 1983). The four tones were chosen to represent the frequencies contained in the male advertisement call. It remains unclear whether estradiol induces a general elevation in the response of the auditory system or an elevation limited to the spectral band of the advertisement call. This distinction is important because a selective effect on the processing of communication signals would suggest an enhancement in the filtering properties of the auditory system. In contrast to estradiol, elevated testosterone levels are

associated with a reduction in sensitivity to pure-tone stimuli in female *H. cinerea* (Miranda & Wilczynski, submitted, chapter two this dissertation). Multiunit thresholds are significantly higher in testosterone-treated females at frequencies corresponding to the spectral bands of the male advertisement call but not at frequencies outside the bands. The responses of auditory systems are not linear across stimulus amplitudes (Moore, 2003) and close-range communication in *H. cinerea* occurs well above the threshold to detect signals (Gerhardt, 1974). For these reasons it is unknown whether frequency-specific testosterone modulation exists at levels consistent with close-range communication.

Reproductive hormones also may influence the auditory system in a stimulus-specific manner. A neuron's responses to pure tones, noise bursts and conspecific advertisement calls are not related linearly (Eggermont et al., 1983; Rose & Capranica, 1983; Rose & Capranica, 1985; Theunissen et al., 2000; Woolley et al., 2006) and individual differences in the neural response may not be universal across stimuli. Evidence from sex differences in *H. cinerea* support the hypothesis that sex differences in auditory processing are stimulus-dependent (Miranda & Wilczynski, submitted, chapter two this dissertation). Males and females do not show differences in multiunit thresholds to pure-tone stimuli within the spectral bands of the male advertisement call, but females do have significantly lower thresholds to a naturally recorded advertisement call.

Spontaneous neural activity in the auditory system also may be modulated by reproductive hormones. Spontaneous activity has recently received increased attention as an important mechanism in sensory processing (Moss et al., 2004). Seasonal differences in the number of spontaneously active cells found in the TS of *Bombina bombina* suggest

that reproductive hormones play a role (Walkowiak, 1980). During the breeding season, the number of spontaneously active cells encountered was greater than in the non-breeding period. The mechanisms of this difference remain unclear but if reproductive hormones are involved, we expect that elevated estradiol and testosterone levels would be associated with increased spontaneous activity. In a previous study, during the breeding season *H. cinerea* females in different reproductive conditions did not differ in spontaneous activity, although it is still unknown whether a change occurs over the longer time span between breeding and non-breeding seasons. Additionally, when quantifying neural responses to auditory stimuli, it is important to know whether group differences are due to underlying differences in spontaneous activity.

In this study we tested whether activation of the hypothalamic-pituitary-goandal (HPG) axis influences female *H. cinerea* multiunit response strengths to auditory stimuli. To activate the HPG axis, we injected females with human chorionic gonadotropin (hCG), a luteinizing hormone (LH) receptor ligand. hCG activates gonadal steroid production (Lutz et al., 2001; Lynch et al., 2006; Wu et al., 2001) and influences communication behavior in male and female anurans (Kelley, 1982; Lynch et al., 2006; Wetzel & Kelley, 1983; Yang et al., 2007). hCG also acts at central nervous system targets directly to influence communication behavior (Yang et al., 2007). In the current study we also tested whether testosterone treatment alone modulates auditory response strengths. For each of these experiments we addressed whether hormone influences were frequency-specific and stimulus-specific. Lastly, we tested whether these hormone manipulations influenced spontaneous neural activity in the TS.

MATERIALS AND METHODS

Animal care

We purchased adult female green treefrogs (*Hyla cinerea)* from Charles Sullivan Co. (Nashville, TN) and housed them in small groups of six animals per 10 gallon aquarium for at least two weeks to acclimate to lab conditions. After hormone manipulation, animals were housed individually in 12"W x 8"D x 7.5"H aquaria. We fed the frogs crickets *ad libitum* and provided water in a bowl inside each aquarium. Environmental conditions were 23ºC and 14:10 light:dark cycle to mimic breeding season conditions. All procedures were performed in accordance with a protocol approved by The University of Texas at Austin Institutional Animal Care and Use Committee.

Hormone manipulation - hCG

hCG was administered through subcutaneous injection. To determine the proper dose, we injected animals with either 0, 10, 100, 500, or 1000 IU of hCG (Sigma) (Figure 14a) and measured estradiol levels using enzyme immunoassay (EIA). Additionally, to determine the time course of hCG effects, we injected eight animals with 1000 IU of hCG and determined estradiol levels at 24, 48, 72 and 96 hours post injection (Figure 14b). Estradiol levels returned to baseline within 72 hours after injection.

For the experimental conditions, females were given multiple injections, optimized to keep estradiol levels chronically elevated as in other steroid manipulation studies. For example, testosterone treatment in female midshipman fish (*Porichthys notatus*) did not significantly increase phase locking in eighth nerve fibers until after 14 days of treatment (Sisneros, Forlano, Deitcher et al., 2004). Additionally, testosterone

treatment for 14 days in female *H. cinerea* increased auditory midbrain multiunit thresholds in response to pure tones that correspond to the male advertisement calls (Miranda & Wilczynski, submitted, chapter two this dissertation). In the current study, females were injected with 500 IU of hCG every 48 hours for a total of four injections over seven days. We injected animals on day one, three, five and seven. Surgery to prepare for the electrophysiology recording took place on day six. Control animals were injected with saline on the same schedule. Electrophysiology recordings took place on the eighth day after the first injection and we collected blood plasma immediately after the recording. Final circulating estradiol levels were (mean ± SE) 0.9669 ± 0.12 ng/ml (n=6) for saline-injected animals and 22.01 ± 6.05 ng/ml (n=5) for hCG injected animals. One of the five females injected with hCG released eggs within 24 hours of the fourth injection.

Hormone manipulation - Testosterone

For the surgical implantation of testosterone, we anesthetized animals by immersion in 0.1% MS-222 buffered with sodium bicarbonate and made a small dorsal cutaneous incision. All individuals were left gonadaly intact and received subcutaneous implants with Silastic© capsules (1.47mm i.d. x 1.96mm o.d. x 5mm total length) either filled with testosterone (n=8) or empty (n=7). These implants were effective in elevating plasma testosterone in previous studies in this species (Burmeister & Wilczynski, 2001; Miranda & Wilczynski, submitted, chapter two this dissertation). We sealed the incision with Vetbond (World Precision Instruments, Sarasota, FL) and placed each animal in its own holding aquarium for 10-12 days. Following the collection of electrophysiological

data we euthanized the animal by anesthetization in 0.2% MS-222 and then rapid decapitation. We then immediately collected a blood sample for hormone analysis. Enzyme immunoassay verified that testosterone implants increased plasma androgen levels consistent with the previous studies in this species. For some samples, testosterone levels were higher than the reliable range of the assay kit but all were at least 375 ng/ml in this study. Testosterone levels in control females (empty implants) were 4.7 ± 0.58 ng/ml.

Hormone analysis

We measured estradiol levels in plasma from hCG-injected females and testosterone from testosterone-treated females. General methods for the EIA procedure have been described previously (Lynch et al., 2006). We assayed ether-extracted plasma using EIA kits (Cayman Chemical, Ann Arbor, MI) for estradiol and testosterone. For estradiol and testosterone respectively, mean recoveries were 24.88 ± 0.67% and 66.95 ± 1.32%, and intraassay coefficients of variation were 19.5% and 9.8%, respectively.

Neurophysiology

All methods for animal preparation, auditory stimuli, neurophysiology and data analysis were previously described (Miranda, Chapter three this dissertation). We measured response strengths to noise bursts and naturally recorded advertisement call stimuli at five stimulus amplitudes each. The magnitude of a response to a given stimulus was calculated as response strength normalized to baseline spontaneous activity. Response strength was defined as a *z*-score. The *z*-score subtracts the mean number of spikes during the baseline period from the mean number of spikes during the stimulus

presentation and divides that by the standard deviation of that difference over 20 stimulus presentations. The calculation is as follows:

$$z = \frac{\overline{S} - \overline{B}}{\sqrt{\mathrm{Var}(S) - \mathrm{Var}(B) - 2\mathrm{Covar}(S,B)}},$$

The stimuli were presented in the form of three different categories based on spectral bandwidth: 1) a full frequency category was bandpass filtered from 100-5000 Hz to correspond with the known hearing range of *H. cinerea*, 2) an AP frequency category was bandpass filtered from 100-1400 Hz to correspond with the sensitivity of the amphibian papilla and 3) a BP category was filtered from 1600-5000 Hz to correspond with the sensitivity of the basilar papilla. A different z-score was calculated for each animal in response to each stimulus and at each stimulus amplitude.

For the advertisement call stimuli, representative calls from seven individual males were presented. We used principle component analysis to summarize the responses to each stimulus category at each stimulus amplitude. The first component of a principle component analysis was an acceptable descriptor of an animal's combined response to the corresponding stimulus category. For the hCG experiment the first component captured a maximum of 91.29% and a minimum of 44.08% of the variance in response to a given stimulus category with a median of 66.29% (for a representative scree plot see Figure 15a). For the testosterone experiment the first component captured a maximum of 93.53% and a minimum of 43.87% of the variance in responses to a given stimulus category with a median of 74.21% (for a representative scree plot see Figure 15b). Unstandardized component scores were then computed for statistical analysis and graphical representation.

Repeated measures ANOVA with a between-subjects factor of hormone treatment was used to compare groups on their response strengths separately for each of the following categories of stimuli: full noise, AP band-limited noise, BP band-limited noise, full advertisement call, AP band-limited advertisement call and BP band-limited advertisement call. For responses to noise stimuli, the dependent variable was the normalized response strength (z-score, for details see Chapter three methods) which was tested at the five stimulus amplitudes. For responses to the advertisement call stimuli, the dependent variable was the unstandardized principle component score of the response strengths which was also tested at the five stimulus amplitudes. When a significant main effect of hormone treatment was supported, we conducted Bonferroni corrected post-hoc analyses to test the overall between-group comparisons. We used unpaired t-tests to compare groups on spontaneous activity. A p-value for statistical significance was set at $p < 0.05$ for all tests in this study.

RESULTS

hCG

Spontaneous activity

hCG treatment did not significantly influence multiunit spontaneous activity [$t(9)= 0.436$, $p= 0.673$]. The mean spike rate for saline-injected females was 33.1 ± 6.7 spikes/second compared to hCG-injected females with 28.8 ± 7.3 spikes/second (Figure 16a). Spontaneous activity scores did not correlate with z-scores [example: z-scores for response to the full frequency category of the advertisement call at 90 dB SPL, r (11) = -0.342, $p = 0.303$] (Figure 16b).

Response strengths

hCG treatment did not significantly influence multiunit response strengths for any of the stimulus categories presented (Figure 17)(Figure 18).

Testosterone

Spontaneous activity

Testosterone treatment did not significantly influence multiunit spontaneous activity [$t(13)= 1.053$, $p= 0.312$]. The mean spike rate for blank implanted females was 17.5 ± 4.2 spikes/second compared to testosterone implanted females with 11.8 ± 3.6 spikes/second (Figure 19a). Spontaneous activity scores did not correlate with z-scores [example: z-scores for response to the full frequency category of the advertisement call at 90 dB SPL, r (15) = -0.036, p = 0.898] (Figure 19b).

Response strengths

Noise

Testosterone treatment did not significantly influence neural response strengths to the full noise stimulus with no significant interaction between stimulus amplitude and treatment [Wilks' lambda = 0.894, F(4,10) = 0.265, p = 0.894] and no main effect of treatment [Wilks' lambda = 0.740, F(5,9) = 0.631, p = 0.681] (Figure 20a). Testosterone treatment also did not significantly influence neural response strengths to the AP noise stimulus with no significant interaction between stimulus amplitude and treatment [Wilks' lambda = 0.592, F(4,10) = 1.721, p = 0.221] and no main effect of treatment

[Wilks' lambda = 0.522, F(5,9) = 1.650, p = 0.242] (Figure 20b). Testosterone treatment did reduce response strengths to the BP noise stimulus at the higher stimulus amplitudes compared to controls with a significant interaction between stimulus amplitude and treatment [Wilks' lambda = 0.402, F(4,10) = 3.724, p = 0.042] (Figure 20c).

Advertisement call

Testosterone treatment significantly decreased response strengths to the full advertisement call at higher stimulus amplitudes with a significant interaction effect between stimulus amplitude and treatment (Wilks' lambda = .279, F(4,10) = 6.453, p=0.008). For the full advertisement call stimuli, treatment significantly reduced response strengths at 80 dB SPL [F(1, 13)= 6.084, p=0.028] and 90 dB SPL [F(1, 13)= 7.603, p=0.016] (Figure 21a). Testosterone treatment also decreased response strengths to the BP advertisement call stimuli at higher stimulus amplitudes with a significant interaction between stimulus amplitude and treatment (Wilks' lambda = 0.262, F(4,10) = 7.059, p = 0.006). For the BP advertisement call stimuli, treatment significantly reduced response strengths at 90 dB SPL [F(1, 13)= 5.878, p=0.031] and 100 dB SPL [F(1, 13)= 6.031, p=0.029] (Figure 21c). Testosterone treatment also showed a trend toward a decrease in response strengths to the AP advertisement call stimuli with a main effect of treatment approaching statistical significance (Wilks' lambda = 0.392, F(5,9)=2.797, p=0.086) (Figure 21b).

DISCUSSION

Our goal for this study was to determine whether modulation of the HPG axis in femlae *H. cinerea* influenced the neural response in the TS to auditory stimuli at levels consistent with close-range communication. In the first experiment, we stimulated the HPG axis using hCG at a level optimized to chronically elevate estradiol levels. In the second experiment, we implanted females with testosterone. For both experiments we tested whether hormone influences were frequency-dependent based on spectral bands associated with sensitivities in the peripheral auditory system. Additionally, we tested whether hormone influences were stimulus-dependent, based on the behavioral relevance of the auditory stimulus. Our results demonstrate that, in females, testosterone significantly reduces neural responsiveness to the male advertisement call but not to a noise stimulus.

The reduction in multiunit response strengths in testosterone-treated females is consistent with a previous study of neural thresholds in *H. cinerea* (Miranda & Wilczynski, submitted, chapter two this dissertation). In the previous study, testosterone-treated females showed a reduction in sensitivity to pure-tone stimuli at neural threshold for auditory stimulation. Here we also see a reduction in responsiveness to suprathreshold stimuli (80-100 dB SPL) that are consistent with natural levels used during close-range communication in this species (Gerhardt, 1974). In the previous study, sensitivity was only significantly reduced for frequencies within the spectral bands of the advertisement call (Miranda & Wilczynski, submitted, chapter two this dissertation). Similarly, in the current study we find that for stimuli that include both high and low frequency ranges, reductions in response strengths are stimulus specific.

Testosterone treated females show significant reductions in response strengths to the advertisement call stimuli but not band-limited noise. Interestingly, testosterone did not significantly reduce sensitivity in response to a field-recorded male advertisement call in the previous study. This is also consistent with our current results in that the reduction in response strengths to the advertisement call occurred only at the higher stimulus amplitudes but not at the lower. Together the results of these two studies support the hypothesis that an elevation in circulating testosterone selectively reduces the responsiveness of the auditory system to the spectral range of the advertisement call.

When the AP and BP spectral bands were considered separately, response strength reductions in testosterone treated females were stimulus specific in the AP range but not the BP range. The lack of a stimulus specific effect in response to high frequency stimuli may reflect a frequency specific effect of testosterone treatment. This effect could occur at the level of the peripheral auditory system, on the BP organ directly, or in the central processing of high frequency stimuli. Neurophysiological recordings from the eighth nerve could help clarify the origin of this frequency specific effect of testosterone on auditory processing.

A reduction in the neural responsiveness to the male advertisement call may explain, in part, reduced behavioral responses of female anurans during periods of elevated androgens. Female *Scaphiopus couchii* and *Rana esculenta* both have elevated testosterone levels during the preovulatory unmated period (Gobbetti & Zerani, 1999; Harvey et al., 1997). Female *Physalaemus pustulosus* show low levels of receptivity and permissiveness coinciding with high androgen levels during the preovulatory stage. The hormone and behavioral profiles of *H. cinerea* throughout the breeding cycle are

currently unknown and are needed to gain a clear picture of how a hormone-induced reduction in neural responses influences reproductive behavior and mate choice. Reduced responsiveness of the auditory system to close-range communication signals may facilitate the inhibition of mating behavior prior to the maturation of eggs and ovulation

We hypothesized that elevated gonadotropins would increase neural responsivness to auditory stimuli but our results do not support this hypothesis. The lack of an influence of hCG may be due to the timing of the treatment protocol. In this study we elevated hormone levels for eight days using multiple injections of hCG. Activation of the HPG axis by gonadotropins may have shorter term effects on auditory processing that do not remain even with extended elevation of steroid hormones. Two injections of estradiol into the third ventricle of the brain of *Rana pipiens* increased auditory evoked potentials recorded from the torus five hours after the first injection (Yovanof & Feng, 1983). Such an effect may be transient and may not be present when estradiol levels are elevated for a period of days. A single injection of hCG would likely match a more natural pulse of gonadotropin release. An examination of neural responses during this brief period of elevated estradiol and other steroids may provide a more appropriate view of auditory processing during this period of elevated reproductive behavior. Additionally, after oviposition female *H. cinerea* do not respond to the male advertisement call in phonotaxis tests (Gerhardt, 1974) and show reduced neural response strengths in the TS to the low frequency component of the advertisement call (Miranda, chapter three this dissertation). Assessment of auditory processing in the days following a single hCG injection may shed light on the neural mechanisms of these post-ovulatory changes.

Other mechanisms not related to gonadotropin release may influence auditory processing of communication signals in females. First, in the current study hCG induced only one out of five females to oviposit despite elevated estradiol in all. Induction of receptivity associated with oviposition in female *Rana pipiens* requires that eggs pass through the oviducts (Diakow et al., 1988). Similarly, female *Physalaemus pustulosus* that received an hCG injection but did not release eggs did not increase receptive behavior (Lynch et al., 2006). If females in the current study were not in the necessary condition to oviposit or if the hCG injection was not sufficient to induce oviposition, this may explain the lack of an influence on auditory processing. Further investigation is needed to identify the neural mechanism of peripheral stimulation of receptivity. Second, the neuropeptide arginine vasotocin (AVT), homologue to the mammalian arginine vasopressin (AVP), may influence auditory processing through direct action on the central nervous system or through osmoregulation. In female anurans AVT increases receptivity and efficiency of phonotactic behavior (Boyd, 1992, 1994; Diakow, 1978; Diakow & Nemiroff, 1981; Raimondi & Diakow, 1981). AVT peptide, receptors and immunoreactive fibers are present in the TS (Acharjee et al., 2004; Boyd, 1997) and AVT regulates neurosteroid biosynthesis (Do-Rego et al., 2006). AVT also plays a role in osmoregulation and is implicated in the regulation of endolymphatic pressure in the inner ear (Oudar et al., 1991; Takeda et al., 2000). Pathological increases in endolymphatic pressure associated with increased AVP levels can dramatically reduce hearing sensitivity although it remains unclear whether cyclic fluctuations in AVT/AVP influences hearing in a way that would impact behavioral responses to communication stimuli.

We found no support for a role of reproductive hormones in modulating spontaneous activity. A previous study of *Bombina bombina* reported encountering more spontaneously active cells during the breeding season compared to a non-breeding period (Walkowiak, 1980) and another study of *Rana pipiens* reported no such difference in males (Goense & Feng, 2005). In agreement with the current study, a third study using multiunit electrophysiology failed to detect a difference in spontaneous activity between female *H. cinerea* in different reproductive conditions (Miranda, chapter three this dissertation). Multiunit recordings may fail to detect changes in spontaneous activity if, for example, the number of spontaneously active cells increased but the spontaneous spike rate for each cell decreased. Single unit studies of spontaneous activity in *H. cinerea* could help clarify whether reproductive hormones modulate this activity and how it is involved in auditory processing. However, we were able to determine that the significant reduction in response strengths in testosterone-treated females is a stimulus-evoked phenomenon because spontaneous activity scores do not correlate with response strength scores.

This study supports the hypothesis that steroid hormones play a role in modulating auditory processing of communication stimuli in the auditory midbrain. The stimulus-specific nature of hormone influences suggests that they may result in individual differences in mating behavior and perhaps mate choice. Behavioral studies of the influence of testosterone on mate choice are needed to understand how variation in the function of sensory systems might influence the evolution of animal communication.

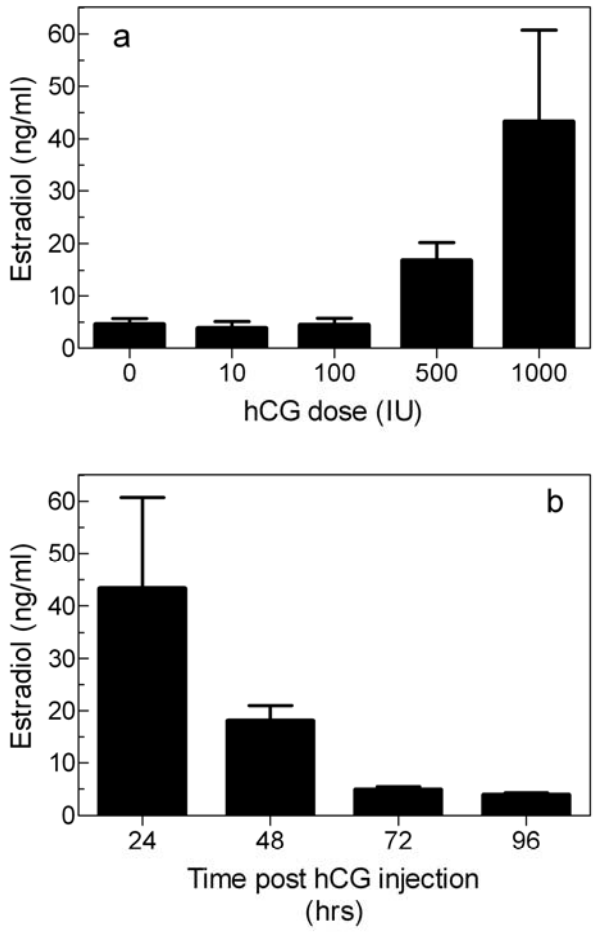

Figure 14. Plasma estradiol levels after a subcutaneous injection of hCG in female *Hyla cinerea*. (a) A dose response curve 24 hours after injection of hCG (n=2 per group, 10 animals total). (b) Estradiol levels over time after a single injection of hCG (n=2 per group, 8 animals total).

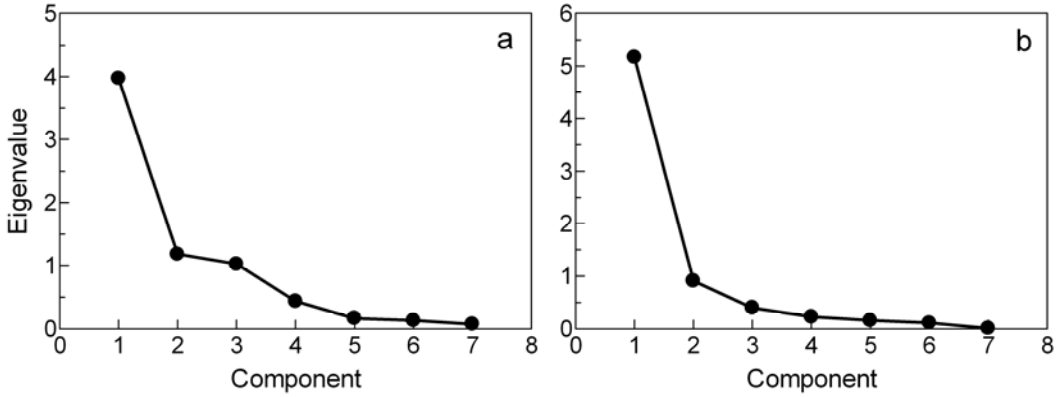

Figure 15. Representative scree plots from principal component analyses of response strengths to the seven advertisement call stimuli presented at 80 dB SPL. (a) animals in the hCG manipulation experiment (n=11). (b) animals in the testosterone manipulation experiment (n=15). Component number one captured a majority of the variance for all stimulus categories. For these examples component number one captured (a) 56.8% and (b) 73.9% of the variance.

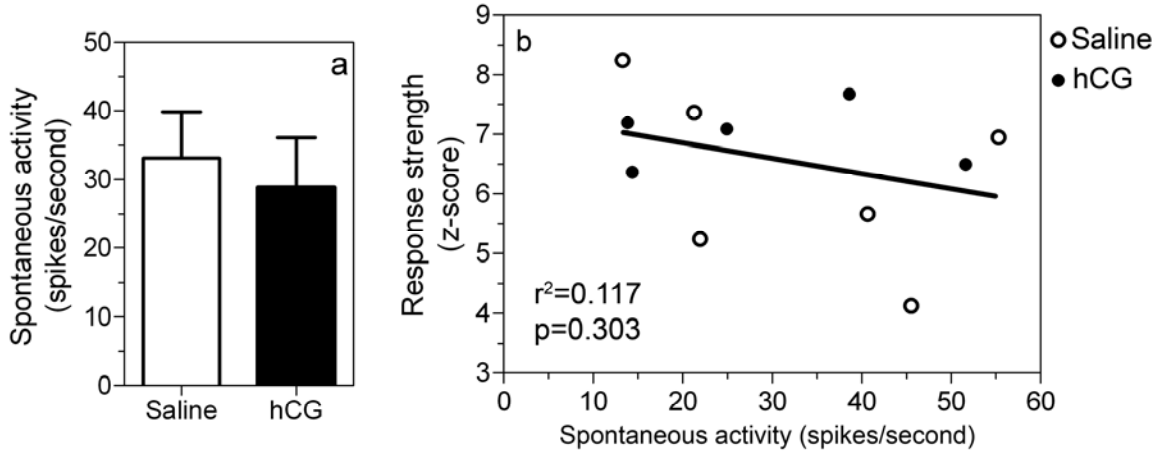

Figure 16. Spontaneous spike rates in the torus semicircularis of female *H. cinerea* treated with hCG. (a) No significant difference between saline and hCG treated females. (b) Spontaneous activity scores do not correlate with response strength scores (open circles: saline, filled circles: hCG). This example plots spontaneous activity scores against z-scores in response to the full frequency category of the advertisement call stimulus presented at 90 dB SPL.

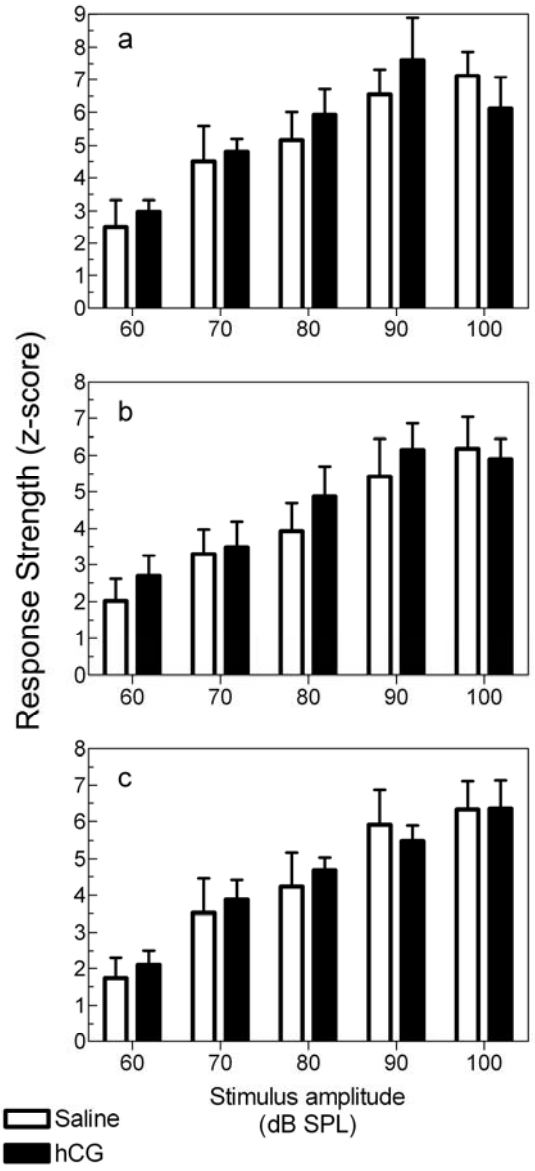

Figure 17. Influence of hCG on torus semicircularis response strengths to noise. The multiunit response is presented as a normalized response strength (z-score, calculated for each individual animal in response to each stimulus type). Each bar represents the mean z-score for the corresponding group at the stimulus amplitude for: (a) noise bandpass filtered to encompass the airborne hearing range of 100-5000 Hz, (b) noise filtered to correspond to the AP range of 100-1400 Hz and (c) the BP range of 1600-5000 Hz. MANOVA revealed no significant difference between hCG and saline treated animals for any of the noise stimuli.

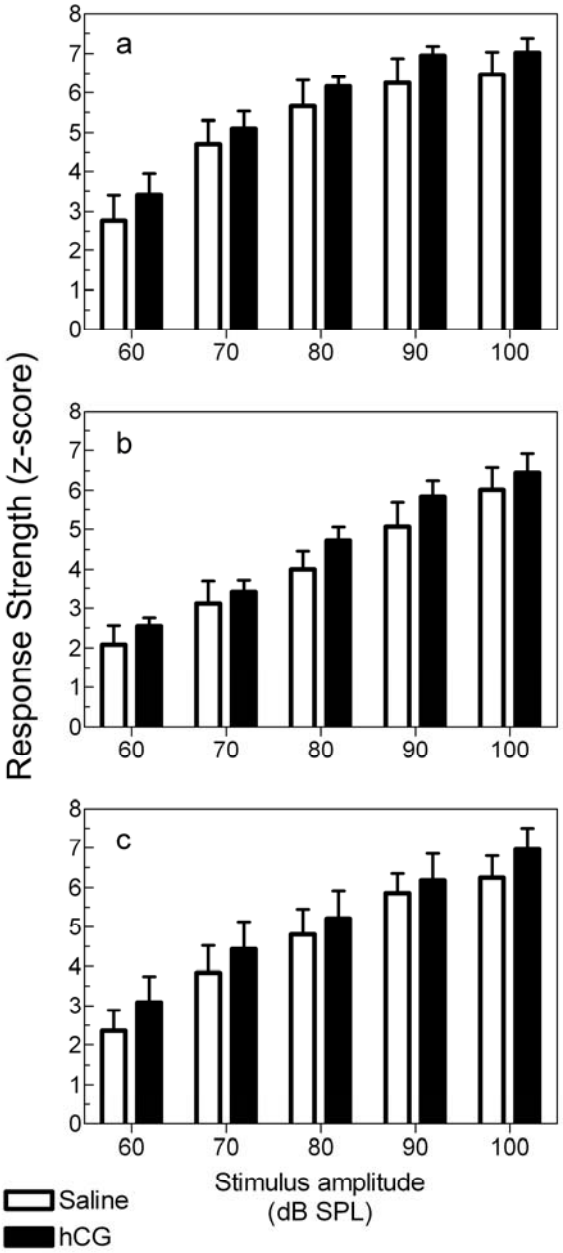

Figure 18. Influence of hCG on torus semicircularis response strengths to the advertisement call. The multiunit response is presented as a normalized response strength (z-score, calculated for each individual animal in response to each stimulus type). Each bar represents the mean unstandardized score from a factor analysis on the response to advertisement calls from seven different male *Hyla cinerea*. (a) noise bandpass filtered to encompass the airborne hearing range of 100-5000 Hz, (b) noise filtered to correspond to the AP range of 100-1400 Hz and (c) the BP range of 1600-5000 Hz. MANOVA revealed no significant difference between hCG and saline treated animals for any of the advertisement call stimuli.

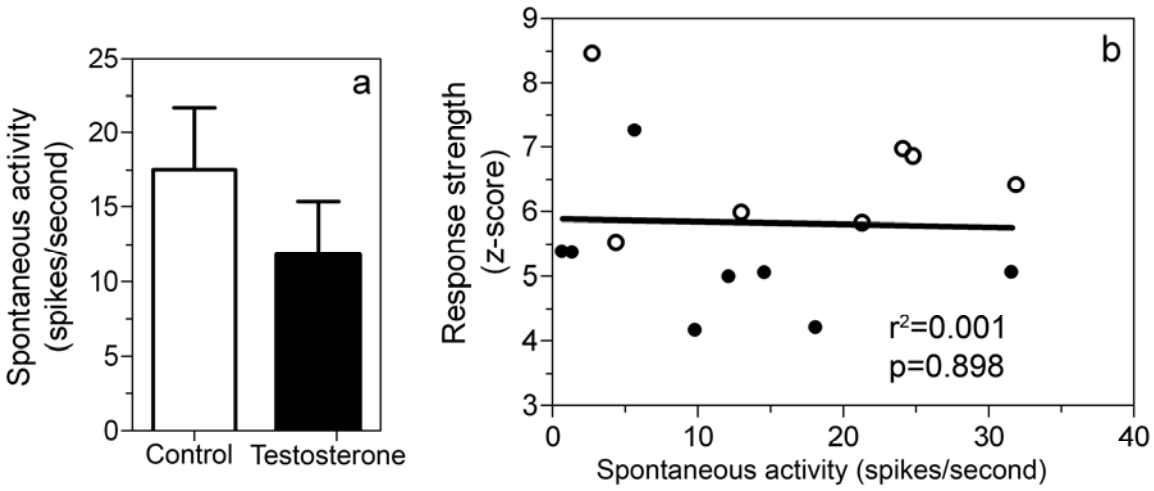

Figure 19. Spontaneous spike rates in the TS of female *H. cinerea* treated with testosterone. (a) No significant difference between control and testosterone-treated females. (b) Spontaneous activity scores do not correlate with response strength scores (open circles: control, filled circles: testosterone). This example plots spontaneous activity scores against z-scores in response to the full frequency category of the advertisement call stimulus presented at 90 dB SPL.

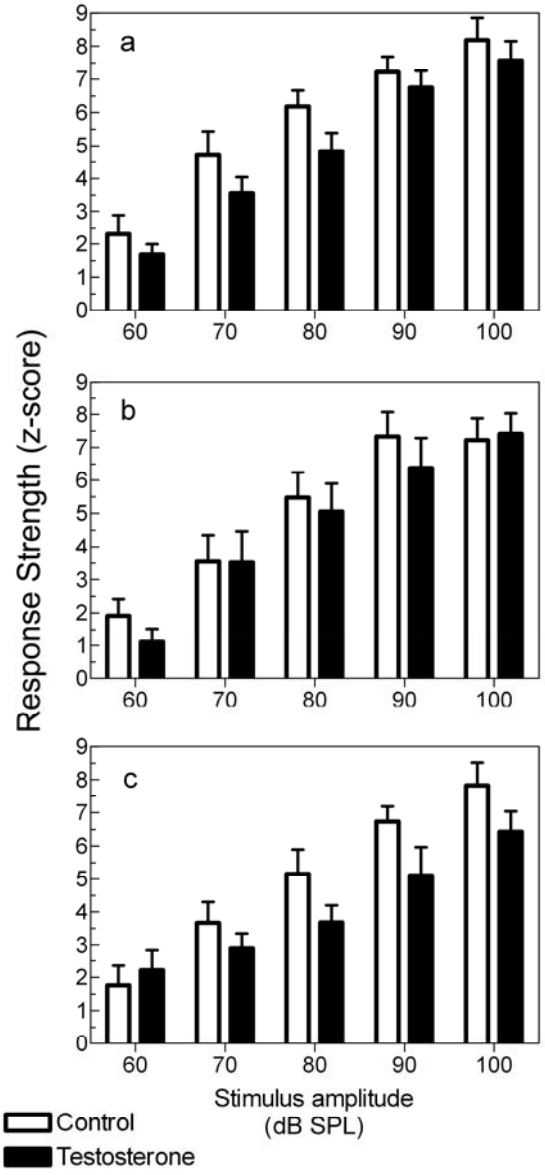

Figure 20. Influence of testosterone on torus semicircularis response strengths to noise. The multiunit response is presented as a normalized response strength (z-score, calculated for each individual animal in response to each stimulus type). Each bar represents the mean z-score for the corresponding group at the stimulus amplitude for in resoonse to (a) noise bandpass filtered to encompass the airborne hearing range of 100-5000 Hz, (b) noise filtered to correspond to the AP range of 100-1400 Hz and (c) the BP range of 1600-5000 Hz. MANOVA revealed no significant difference between hCG and saline treated animals for any of the noise stimuli. Females treated with testosterone did show significant reduction in response strengths in the BP range ($p<0.05$) at higher but not lower stimulus amplitudes.

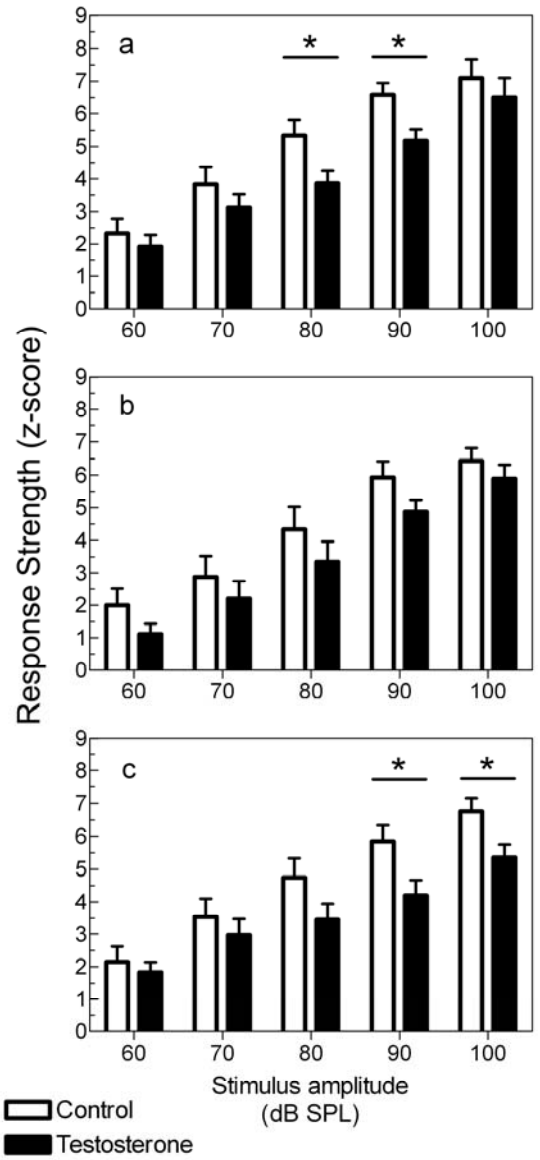

Figure 21. Testosterone reduces torus semicircularis response strengths to the advertisement call. The multiunit response is presented as a normalized response strength (z-score, calculated for each individual animal in response to each stimulus type). Each bar represents the mean unstandardized score from a factor analysis on the response to advertisement calls from seven different male *Hyla cinerea*. Response strengths to advertisement calls (a) bandpass filtered to encompass the airborne hearing range of 100-5000 Hz, (b) filtered to correspond to the AP range of 100-1400 Hz and (c) the BP range of 1600-5000 Hz. MANOVA across stimulus amplitudes is significant in the full range ($p<0.01$) and the BP range ($p<0.05$). Bars with asterisks denote stimulus amplitudes with a significant post-hoc test for testosterone treatment ($p<.05$). Testosterone-treated females also showed a trend toward a decrease in response to the AP range ($p<0.1$).

Chapter 5: Concluding remarks

REPRODUCTIVE HORMONES INFLUCENCE AUDITORY PROCESSING

Research in this dissertation provides strong evidence that reproductive hormones influence auditory processing. First, if reproductive hormones influence auditory processing, males and females would be expected to differ in their neural responses to sound. Experiments on multiunit response thresholds and response strengths demonstrate such differences at the level of the auditory midbrain, the torus semicircularis (TS). Sex differences could be due to developmental differentiation that results in stable response patterns throughout the adult life of the animal. Alternatively, the auditory system may vary in its response to stimuli across different stages of the animal's life as hormones vary. If circulating reproductive hormones affect sensory processing, then auditory midbrain responses are likely to vary with reproductive condition. Female reproductive condition does appear to influence auditory processing as suggested by reduced response strengths in postmated females when compared to unmated females. The prediction is that variation in hormone levels would correspond with differences in midbrain responses to sound because female reproductive condition is associated with fluctuations in gonadal steroid levels. Evidence in this dissertation suggests that circulating androgen levels do influence auditory processing in females by increasing neural response thresholds and decreasing response strengths.

CHARACTERISTICS OF HORMONE EFFECTS SUGGEST INFLUENCES ON COMMUNICATION RELATED AUDITORY PROCESSING

Sex differences

These experiments also suggest that reproductive hormones influence auditory processing in ways that shape the filtering properties of the auditory system for the detection of communication signals. If sex differences influence neural responses to natural stimuli as opposed to a general influence on all auditory responses, then differences could be frequency-dependent. This research provides evidence that sex differences in response thresholds and response strengths as measured in the TS are frequency-dependent. Multiunit audiograms measured from the TS in these and previous studies established that *H. cinerea* have three sensitivity peaks. Sex differences occur only at two of the three peaks, with males having lower thresholds in the lower frequency range corresponding with the AP spectral band. Thresholds are not different at the peak in the high frequency range corresponding with the BP spectral band. Sex differences in response strengths show a similar pattern with females having lower response strengths to stimuli within the AP spectral band. This difference in response strength is dependent on the reproductive condition of the female with postmated females different from males and unmated females. Males and unmated females showed no differences. Females in the threshold study could have been in a postmated-like condition resulting in higher thresholds within the AP spectral band. The dependence of sex differences in response thresholds on female reproductive state requires further attention because reproductive condition was not considered when measuring response thresholds. Additionally, when

considering all of the frequencies of the audiogram together based on whether or not they fall inside or outside the spectral bands of the male advertisement call, females have significantly higher thresholds to frequencies outside the spectral bands. Males and females are not significantly different when considering frequencies inside the spectral bands. These frequency-dependent results suggest that, in a noisy environment, the female auditory system may filter out frequencies that would interfere with the detection and discrimination of the communication signal. Experiments comparing males and females on these tasks are needed to test the behavioral implications of audiogram sex differences.

If sex differences influence auditory processing of communication signals then differences could also be stimulus-dependent. This is particularly possible in the TS, where studies have shown that a portion of the neurons have complex "call detector" properties. For response thresholds, experiments in this dissertation provide evidence that sex differences in response to pure-tone stimuli are unlike sex differences in response to the male advertisement call. Whereas males and females do not differ in thresholds to tones within the male advertisement call spectral bands, females do have significantly lower thresholds than males in response to a natural recording of the advertisement call. Additionally, when males and females do differ in thresholds to pure tones, females have higher thresholds which contrasts with the lower thresholds for the advertisement call. These results suggest that for threshold responses the characteristics of sex differences depend on the type of stimulus that the auditory system is responding to. Future single-unit studies could address the encoding mechanisms that result in stimulus-dependent sex differences observed at the multiunit level. For response strengths, sex differences demonstrated in the AP spectral band do not provide evidence for a stimulus-dependent

sex difference in these studies. Postmated females had significantly reduced response strengths to both noise burst and advertisement call stimuli filtered to only include the AP spectral band. Males, postmated females and unmated females did not differ in response either to the full advertisement call or the full noise burst stimuli. These results suggest that sex differences as measured at the multiunit level are not linear across a range of stimulus amplitudes.

Androgen influences

If androgens influence auditory processing of communication signals, then differences in circulating androgen levels could influence neural responses in a frequency-dependent and stimulus-dependent manner. These experiments provide evidence that androgens increase response thresholds and reduce response strengths in females in a frequency-dependent and stimulus-dependent manner. Females with experimentally increased circulating androgen levels show elevated multiunit thresholds in response to pure-tone stimuli that fall within the spectral bands of the male advertisement call. This effect was most pronounced in response to tones within the BP spectral band. Females showed no evidence of elevated thresholds outside the spectral bands of the advertisement call. Similarly, females with elevated androgen levels showed reduced response strengths to field-recorded male advertisement calls but not to band-limited noise. This effect was significant for the full advertisement call and the call presented with only the BP spectral band. Together these results suggest a selective reduction in responsiveness to spectral components of the advertisement call in females when androgens are high.

LIMITATIONS TO INTERPRETATIONS OF THESE STUDIES

There are some limitations to the interpretation of multiunit responses in the TS that leave several questions to be addressed in future studies. This level of assessment is much like that of evoked potential recordings and measurement of blood flow using fMRI. First, multiunit responses do not provide direct information about neural encoding of auditory stimuli by individual neurons. Multiunit responses do provide an assessment of the combined level of activation of neurons in the TS to a range of stimuli. When sex or hormones significantly influence the response of the auditory system, multiunit responses will show significant differences that may be difficult to detect at the single-unit level of analysis. On the other hand, small or subtle differences in the responses of individual neurons might not be detected in multiunit recordings. For example, the lack of a stimulus-dependent sex difference at higher stimulus amplitudes reported here may be the result of the limitations of multiunit electrophysiological studies. Nonetheless, differences in multiunit responses do strongly suggest that significant differences in single-unit responses do occur, which could then be more precisely understood with single-unit extra- or intra-cellular recordings. Second, neural responses in the TS are not necessarily the result of intrinsic response properties of TS neurons but may be the result of response properties of afferent inputs. For this reason, conclusions from these experiments do not address whether sex or hormone effects emerge first in the TS. Results from experiments in this dissertation do support a role for sex and circulating androgens in influencing auditory processing and this can be seen at the level of the auditory midbrain. Questions remain as to whether hormones influence auditory processing at lower levels of the auditory system.

BEHAVIORAL SPECULATIONS

For sex differences in neural response thresholds, higher thresholds to frequencies outside the bands of the male advertisement call in female *H. cinerea* suggest that, in a noisy environment, the female auditory system may filter out frequencies that would interfere with the detection and discrimination of the communication signal. Broadband noise does influence behavioral responses in female *H. cinerea* (Ehret and Gerhardt, 1980). Additionally, behavioral evidence in females of another Hylid species, *H. ebraccata*, suggests that the natural noise of a chorus does indeed impair detection and discrimination (Wollerman, 1999; Wollerman and Wiley, 2002). Experiments comparing males and females on these tasks are needed to test the behavioral implications of audiogram sex differences.

The reduction of neural responsiveness to stimuli within the AP spectral band in postmated females may have behavioral effects associated with female mating behavior. The *H. cinerea* male advertisement call has two spectral peaks, one falling within the AP spectral band and the other falling within the BP band. Females are behaviorally sensitive to the relative amplitudes of the spectral peaks of the advertisement call (Gerhardt, 1974). In two-alternative choice tests, a synthetic call with a relative amplitude difference of as little as 10 dB SPL between the low and high spectral peaks rendered the call less attractive compared to calls with smaller differences. Gravid females fail to respond to the male advertisement call after oviposition (Gerhardt, 1974). A significant reduction in the neural responsiveness to one of the spectral bands may result in a perceptual shift that renders a previously attractive call unattractive to a female that has already mated. A detailed behavioral analysis with females in different reproductive conditions is needed to determine whether a shift in the neural

representation of this social signal would result in changes in recognition, discrimination or detection.

A reduction in the neural responsiveness to the male advertisement call may explain, in part, reduced behavioral responses of female anurans during periods of elevated androgens. Female *Scaphiopus couchii* and *Rana esculenta* both have elevated testosterone levels during the preovulatory unmated period (Gobbetti & Zerani, 1999; Harvey et al., 1997). Female *Physalaemus pustulosus* show low levels of receptivity and permissiveness coinciding with high androgen levels during the preovulatory stage. The hormone and behavioral profiles of *H. cinerea* throughout the breeding cycle are currently unknown and are needed to gain a clear picture of how a hormone-induced reduction in neural responses influences reproductive behavior and mate choice. Reduced responsiveness of the auditory system to close-range communication signals may facilitate the inhibition of mating behavior prior to the maturation of eggs and ovulation. Androgens are generally viewed as activating reproductive behavior in vertebrates. Results from these studies suggest that androgens could also reduce auditory evoked behavioral responses through action on the auditory system of females.

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Vita

Jason Anthony Miranda was born in Phoenix, Arizona on May 16, 1978, the son of Amy Ann Miranda and Ruben Miranda. After completing his work at Centennial High School, Peoria, Arizona, in 1996, he entered Glendale Community College in Glendale, Arizona. He then transferred to Arizona State University West in Glendale, Arizona. He received the degree of Bachelor of Science from the Barrett Honors College at Arizona State University in May 2001. In August 2001 he entered the Graduate School of the University of Texas.

Permanent address: 1253R Church Street, Decatur, Georgia 30030

This dissertation was typed by the author.